

Remarks

By way of this amendment, claims 1, 11, 18, and 42 are amended and claims 10, 17, and 62-65 are canceled. Upon entry, claims 1, 11-16, 18, 21, and 42-45 will be pending.

Claim Rejections under 35 U.S.C. § 102(b)

The Examiner rejected claims 1-2, 7-9, and 19-20 under 35 U.S.C. § 102(b) as being anticipated by Kretz (U.S. Patent No. 6,110,719). Applicants respectfully traverse.

The Examiner contends that Kretz discloses the claimed composition at col. 3, lines 32-40. The Examiner contends that Kretz discloses a modified compound at col. 9, lines 27-35; col. 10 lines 24-37; col. 13 lines 20-24, and col. 14, lines 23-25. Applicants respectfully disagree and assert that the Examiner is importing an unreasonably broad definition to the term “modified”.

However, to expedite prosecution, Applicants amended claims 1 and 16 to specify that the modified compound comprises nucleic acid comprising an amine group, a sulfhydryl group, or mixture thereof. Applicants amended claim 1 to include the features detailed by claim 62 and claim 16 to include the features of claim 63. Claims 62 and 63 were not subject to this rejection.

Based on at least the reason outlined above, Applicants submit that Kretz does not anticipate claims 1, 14, or 16 or any claim depending therefrom. Applicants respectfully request that the rejection be withdrawn.

Claim Rejections under 35 U.S.C. § 103(a)

Claims 1-2 and 7-21: Kreft, et al. and Veraart, et al.

The Examiner rejected claims 1, 10-18, and 21 under 35 U.S.C. § 103(a) as being unpatentable over Kreft, et al. and Veraart, et al. Applicants respectfully traverse.

To establish *prima facie* obviousness, three basic criteria must be met. “First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference

or combine reference teachings. Second, there must be a reasonable expectation of success. Third, the prior art reference (or references when combined) must teach or suggest all the claim limitations.” (See MPEP 2142).

The Examiner contends that Alberts, et al. illustrate that it has been long known that certain nucleic acid bases comprise amine groups. While Applicants do not disagree with the Examiner’s statement, Applicants do traverse that Alberts, et al. teaches nucleic acids modified as contemplated by the claims.

The Examiner is importing unreasonable breadth to term the “modified” and “amine group”. The Federal Circuit set forth the standard by which the USPTO is to construe claims. Section 2111 of the MPEP quotes the language of the Federal Circuit: “[t]he Patent and Trademark Office (“PTO”) determines the scope of the claims in patent applications not solely on the basis of the claim language, but upon giving claim terms their broadest reasonable construction **in light of the specification as it would be interpreted by one of ordinary skill in the art**”. See MPEP § 2111 citing *Phillips v. AWH Corp.*, 415 F.3d 1303, 1321 (Fed. Cir. 2005).

Applicants appreciate that limitations from the specification cannot be imported into the claims. See *In re Van Guens*, 988 F.2d 1181 (Fed. Cir. 1993). However, there is a distinction between using the specification to define a term and impermissible importation of limitations. Indeed, the Federal Circuit directs that “[c]laims are not to be read in a vacuum, and limitations therein are to be **interpreted in light of the specification** in giving them their broadest reasonable interpretation.” See MPEP § 2111.01 citing *In Re Marosi*, F.2d 799 (Fed. Cir. 1983) (emphasis added).

Applicants describe the term “modification” at page 12, lines 17-18 where it is stated that the modification is to add a functional group that aids in immobilization. Again, modification is detailed at page 12, lines 26-31, where specific reactive groups (amine or sulfhydryls) are discussed. The Examiner contends that the presence of amine groups in certain bases is the equivalent to the modification by the addition of reactive amine groups described the specification. Applicants respectfully disagree.

The claims define the compound as “modified” with an amine group. The amine group of nucleic acid bases is not a modification as defined by the specification or any

other source. Rather, the amine referenced by the Examiner, is simply a part of the molecule. Those skilled in the art, understand the term “amine modified” since this technology has been used for some time as evidenced by the Beaucage paper, a copy of which is included herewith. Beaucage describes the chemistry of attaching oligonucleotides to a surface. At page 1214, Beaucage outline amine modification of DNA. Likewise, the Guo, et al. and Rogers, et al. references cited by the Examiner also demonstrate that the modifications of nucleic acid featured in the claims were well known in the art.

The specification does not support the Examiner’s definition of the term “modified nucleic acid comprising an amine group....”. The specification describes modification as follows “The biomolecule can optionally be functionalized or modified by any variety of know methods. For example, during synthesis, biomolecules, such as oligonucleotides or nucleic acids, can be prepared with functional groups such as amines or sulfhydryl groups in order to be reactive to NOS groups in the polymer composition.” (page 12, lines 26-29 and pages 17-25). This makes clear that the modification involves the user to make a specific addition of an amine group (or sulfhydryl group) that is reactable with the NOS group. One skilled in the art, reading the specification would conclude that the modified compound comprising a nucleic acid that comprises an amine group references an amine group that is added by the user and not the amine group present in the molecule “naturally”.

Applying the standards promulgated by the Federal Circuit and outlined in the MPEP, one skilled in the art would conclude that the modification of nucleic acid does not encompass the amine group naturally present in the oligonucleotide molecule. Therefore, the combination of cited references fails to teach or suggest each and every element of the claims subject to this rejection. Applicants respectfully request that the rejection be withdrawn.

Claims 1 and 14-15: Kretz in view of Sambrook, et al.

The Examiner rejected claims 1 and 14-15 under 35 U.S.C. § 103(a) as being unpatentable over Kretz and Sambrook, et al. Applicants respectfully traverse.

The combined teaching of Kretz and Sambrook, et al. fail to disclose each and every element of the claims. Claim 1 includes a feature directed to nucleic acids modified with an amine group, a sulfhydryl group, or mixture thereof.

Neither Kretz not Sambrook, et al. teach or suggest a modified nucleic acid as defined by the claims. Therefore, Applicants submit that the claims are not obvious in light of the combination of Kretz and Sambrook, et al. and respectfully request that rejection be withdrawn.

Claims 42-45: Guo, et al. and Veraart, et al.

The Examiner rejected claims 42-45, and 65 under 35 U.S.C. § 103(a) as being unpatentable over the combination of Guo, et al. and Veraart, et al. Applicants respectfully traverse.

The Examiner concludes that “[a]n artisan would be motivated to include phytic acid in a method of spotting organic compositions such as nucleic acids because phytic acid has a relatively large ionic strength as compared with its concentration. . . and the art recognizes that increasing ionic strength can reduce non-specific absorption on a support”. Applicants respectfully disagree.

As explained in the specification at page 14 lines 1-25, the invention is directed to improving spot morphology. More specifically, the invention is directed to reducing the so-called “doughnut” effect so as to produce spots with decreased size. The doughnut effect is the result of the concentration of spotted compound or signal at the perimeter of the spot. Thus, one can imagine that during the drying process the compound migrates to the edges of the spot of buffer such that when the buffer dries a increased concentration of spotted compound is deposited around the perimeter.

Veraart, et al. only discuss the usefulness of phytate as a buffer additive to reduce non-specific absorption of proteins on capillary walls. The mechanics of the doughnut effect illustrate that solutions directed to reducing non-specific absorption do not bear on the problem of reducing spot size. Therefore, an artisan reading Veraart et al. would not conclude that there might be a benefit derived from the addition of phytic acid to an array spotting buffer. Thus, one skilled in the art faced with the problem of DNA doughnuts would not be motivated by the teachings of Veraart et al. to employ phytate in a method

of forming spots of nucleic acid since non-specific binding is not an issue in the process of spotting.

The combination of Guo, et al. and Veraart, et al. do not teach or suggest the use of phytate to produce a nucleic acid array. Therefore, the claims are not obvious in light of the combination of references. Applicants respectfully request that the rejection be withdrawn.

Claims 42 and 65: Guo, et al., Veraart, et al. and Rogers, et al.

The Examiner rejected claims 42 and 65 under 35 U.S.C. § 103(a) as unpatentable over Guo, et al., Veraart, et al., and Rogers, et al. Applicants respectfully traverse.

Rogers, et al. disclose the use sulfur modifications of oligonucleotides for the purpose of attaching the oligonucleotide to a surface. However, Rogers, et al. do not cure the deficiencies of Guo, et al. and Veraart, et al. outlined above. Therefore, the claims are not obvious in light of the combination Guo, et al., Rogers, et al. and Veraart, et al. Applicants respectfully request that the rejection be withdrawn.

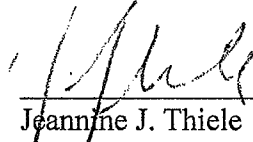
Claims 42 and 45: Guo, et al., Veraart, et al., Rogers, et al. and Lemieux, et al.

Lemieux, et al. teach pin piezoelectric spotting. However, Lemieux, et al. do not cure the deficiencies of Guo, et al., Veraart, et al., and Rogers, et al. outlined above. Therefore, the claims are not obvious in light of the combination of the references. Applicants respectfully request that the rejection be withdrawn.

In view of the foregoing, it is submitted that each of the pending claims are in condition for allowance. Favorable consideration and prompt allowance of the application are respectfully requested.

If the Examiner feels that prosecution of the present application can be materially advanced by a telephonic interview, the undersigned would welcome a call at the number listed below.

Respectfully submitted,



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Strategies in the Preparation of DNA Oligonucleotide Arrays for Diagnostic Applications

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Abstract: This report emphasizes the interfacial chemistry that is required to ensure proper attachment of oligonucleotides onto the surface of microarrays. For example, strategies for the covalent attachment of pre-synthesized oligonucleotides to glass slides, gold films, polyacrylamide gel pads, polypyrrole films, and optical fibers are surveyed in an attempt to better define the parameters for optimal formation and detection of DNA hybrids. These parameters include among others, the nature and length of the linkers attaching oligonucleotides to the arrays, and the surface density of oligonucleotides required for unhindered hybridization with DNA targets. Sensitive detection methods such as the use of light-scattering techniques, molecular beacons, surface plasmon resonance, attenuated total internal reflection-FTIR, and the evanescent field excitation of fluorescence from surface-bound fluorophores have been developed to study the kinetics and specificity of hybridization events. Finally, the synthesis of oligonucleotides directly on glass surfaces and polypropylene sheets has been investigated to enable DNA sequencing by hybridization and achieve oligonucleotide densities of *ca.* 10^6 sequences per cm^2 on DNA chips.

INTRODUCTION

Given that only a fraction of all encoded genes is expressed in any given cell, the timing and levels of gene expression govern cellular development, differentiation, and physiology. A method for monitoring in parallel the expression levels of several genes involves the use of DNA microarrays (or DNA chips). In essence, DNA microarrays consist of a large number of DNA probes immobilized in a known arrangement on solid surfaces such as, for example, a glass slide. The immobilized DNA probes are then incubated in a solution of labeled nucleic acid targets which may be mRNA, genomic DNA, or cDNA from a tissue of interest. The presence and abundance of specific target sequences within the sample are indicated by the intensity of the hybridization signal at the corresponding probes sites, and thus provide a sensitive and high throughput method for gene expression analysis. DNA chip technologies are distinguished by the sizes of arrayed DNA probes, the type of surface and methods used for arraying the probes, the chemistries employed for immobilizing DNA on the chips, and by the hybridization and detection methods. The two major strategies that are widely applied to DNA chip technologies relate to the cDNA array format [1] and the *in situ* synthesized oligonucleotide array format [2].

cDNA Microarrays are generated by high-speed robotic deposition of PCR-amplified cDNA (or genomic) clones on glass slides coated with either poly-L-lysine [1,3,4] or aminopropyltri-ethoxysilane. Microarrays of up to 10,000

clones can be generated [5]. The non-covalent charge interactions between the negatively charged phosphodiester groups of the cDNAs and the positively charged amino functions of, for example, surface-bound lysine side-chains do not form an irreversible attachment of the cDNAs to the glass plate, and result in decreased sensitivity due to loss of cDNAs from the glass surface. In addition, electrostatic interactions between the cDNAs and the glass slide reduce the conformational freedom of the bound cDNAs and, hence, their affinity and specificity for complementary molecules in solution.

Fluorescent cDNA probes are generated from control and test RNA samples in single-round reverse transcription in the presence of fluorescently labeled dUTPs such as Cy3-dUTP and Cy5-dUTP. The strength of this approach resides in the ability to label RNAs from control and treated samples with different fluorescent markers. This method allows the simultaneous hybridization and detection of both populations on one microarray, and eliminates the need to control for hybridization between arrays. This strategy has been successfully applied to the study of *Arabidopsis thaliana* RNA [1], yeast genomic DNA [3], tumorigenic versus non-tumorigenic human tumor cell lines [6], human T-cells [7], yeast RNA [8], and human inflammatory disease-related genes [9]. Although the cDNA microarray format has the inherent advantage of being parallel with a direct and rapid readout of hybridization results, a major disadvantage of this technology is that in order to monitor many genes a large number of cDNAs or PCR products must be prepared, purified, quantitated, and catalogued; these operations can be labor intensive.

The *in situ* synthesized oligonucleotide array format originated from the combination of oligonucleotide synthesis

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and photolithographic computer chip technology to generate DNA chips displaying up to 400,000 oligonucleotides. These can probe up to 9,000 genes on a glass surface of 1.6 cm². The photolithographic approach to the preparation of DNA arrays requires irradiation with ultraviolet light through holes of photolithographic masks on the surface of the chip to remove, in a stepwise manner, photolabile protecting groups from the growing oligonucleotide chains [10]. Given that the stepwise synthesis yield is approximately 85-95%, oligonucleotides no longer than 25 bases can be synthesized. These relatively short oligonucleotides show limited sensitivity and specificity although specificity can be compensated by synthesizing many (ca. 20) oligonucleotides for each gene to be analyzed [11,12]. Furthermore, each oligonucleotide that matches a gene sequence is paired with a second mismatched oligonucleotide that differs only in one centrally located nucleotide. This strategy permits to sort out, during data analysis, spurious hybridization by excluding oligonucleotides that fail to hybridize more strongly than the corresponding mismatched oligonucleotides, and improves signal quantitation. This strategy also allows low-intensity hybridization patterns from, for example, rare RNAs to be sensitively and accurately recognized [12].

Sample preparation involves the generation of double-stranded cDNAs from cellular polyadenylated RNAs followed by antisense RNA synthesis in an *in vitro* transcription reaction with biotinylated or fluorescently labeled nucleoside triphosphates. The RNA probes are then fragmented to facilitate hybridization. Hybrids are visualized either directly or upon incubation with fluorescently labeled streptavidin [11,12]. DNA oligonucleotide microarrays have been applied to the mapping of genomic library clones [13], *de novo* sequencing by hybridization [14], and to evolutionary sequence comparisons of the human hereditary breast and ovarian cancer gene *BRCA1* [15]. In addition, mutations in the cystic fibrosis [16] and *BRCA1* [17] genes, and polymorphisms in the HIV-1 clade B protease gene [18] have been detected by DNA oligonucleotide chips. This technology has been further applied to the identification of genes differentially regulated by interferon α , β or γ [19], and the detection of single nucleotide polymorphisms in the human [20] and yeast [21] genomes [22]. A limitation of the DNA oligonucleotide chip technology, however, is that knowledge of the DNA sequence to be studied is absolutely required to produce the DNA chips.

The usefulness of the two DNA microarray formats described above for measuring expression of a large number of genes has been spectacularly demonstrated with the expression analysis of all 6,000 genes of the bakers' yeast *Saccharomyces cerevisiae* grown under different conditions [4,11]. The data indicate that it is possible to group genes together on the basis of similar patterns of expression and, importantly, that functional relationships can be predicted from these patterns. One of the most promising applications of DNA microarrays is the identification of all those genes changing expression levels when a specific gene is inactivated. Such information would be invaluable to one interested in transcription factors as it should help unravel their roles in cellular physiology.

Because of their relative simplicity, DNA chips are particularly suited for studying the expression patterns of genes involved in disease progression, and the identification of point mutations and single nucleotide polymorphisms in genomic DNA that predispose human to diseases. Although conceptually simple, hybridization of nucleic acid targets to DNA oligonucleotide arrays depends on a number of parameters. These include: (i) the chemical and physical properties of the support surface; (ii) the nature and length of linkers tethering the oligonucleotide probes to the solid surface; (iii) the attachment density of the probes on the surface; (iv) the length and type (single- stranded versus double-stranded) of target DNA molecules; (v) the sequence and length of the DNA oligonucleotide probes; and (vi) the hybridization and washing conditions [23]. Considering the importance of these parameters on the performance of DNA microarrays, this review will focus on the various types of interfacial designs that will permit either the attachment of pre-synthesized oligonucleotides or direct synthesis of DNA oligomers on the arrays. New methods for detecting interfacial DNA hybridization events will also be reviewed. Because of the impressive technical advances in microarray development, this report cannot be comprehensive and, consequently, many excellent reports have regrettably been omitted. However, this review attempts to delineate the salient features and trends in microarray technology, along with the basic research issues and technical obstacles that remain to be addressed before benefiting from the outstanding bioanalytical potential of this rapidly evolving technology.

The first section of this report deals with the covalent immobilization of pre-synthesized oligonucleotides on planar glass surfaces.

COVALENT ATTACHMENT OF DNA OLIGONUCLEOTIDES ON GLASS SLIDES

Glass slides are readily available, inexpensive, and possess a relatively homogenous chemical surface with well-studied properties. Glass is also amenable to chemical modifications *via* versatile and well-known silanization chemistries. The coupling chemistry for linking oligonucleotides to silanized glass surfaces must, however, be developed and satisfy the following criteria: (i) the attachment must be chemically stable; (ii) the linker should be lengthy enough to eliminate undesired steric interferences from the glass surface; (iii) the linker should also be hydrophilic to ensure complete solubility in aqueous solution and; (iv) the chemical attachment should not produce non-specific binding to the glass surface.

Prior to functionalization, glass microscope slides are usually activated by treatment with a strong acid (1 N HNO₃) [23]. The slides are then immersed in a solution of 3-glycidioxypropyl trimethoxysilane, *N,N*-diisopropylethylamine, and anhydrous xylene (8:1:24 v/v) at 80 °C [23-25] (see Fig. (1)). Synthetic oligonucleotides (9-mers) were prepared according to standard phosphoramidite chemistry [26-28], and functionalized to contain an amino group at either the 5'- or 3'- terminus and a triethylene glycol phosphoryl spacer to provide a variable distance between the glass surface and the oligonucleotides [23]. An

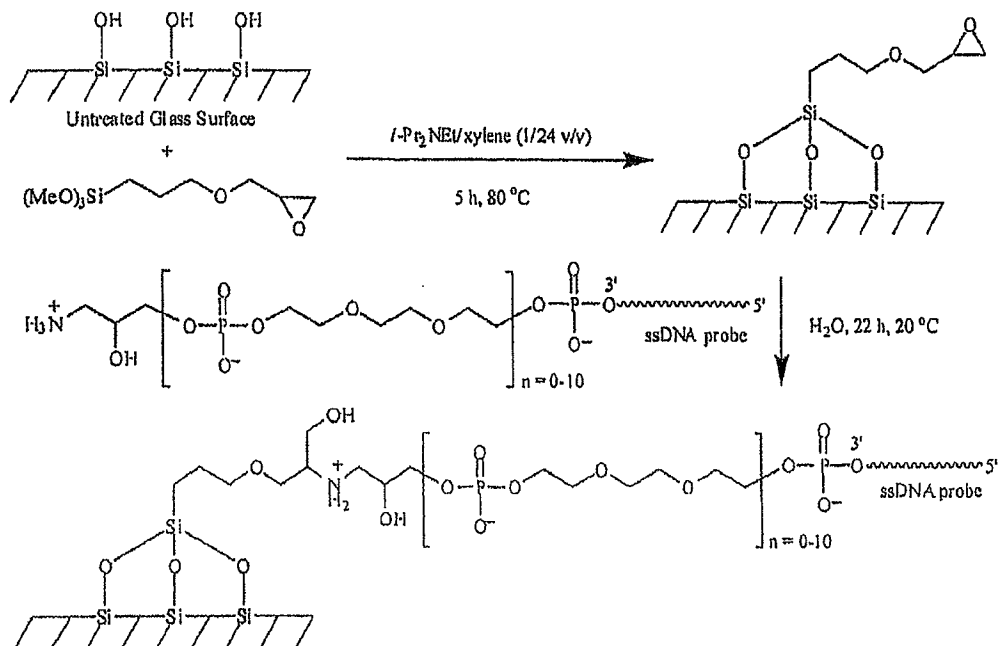


Fig. (1). Covalent attachment of 3'-aminated oligonucleotides carrying triethylene glycol spacers to epoxysilanized glass slides.

aqueous solution of a functionalized oligonucleotide (100 μM) was then applied (200 nL) to the epoxysilanized glass slide (Fig. (1)) by the use of a robotic workstation, and incubated for 22 h at 20 °C or 37 °C to afford an oligonucleotide attachment density of *ca.* 500 fmoles over a 1.5 mm diameter spot (10^{10} - 10^{11} probes/ mm^2). Parenthetically, a similar strategy has been adopted for the sensitive and rapid detection of nucleic acid hybridization on the surface of a charge-coupled device [29]. Thus, hybridization of glass-tethered 9-mers with PCR ^{32}P -labelled DNA fragments led to the capture of 1.3 kb target strands. Hybridization of the longest targets improved with increased spacer length between the glass surface and the oligonucleotide probes. Glass-tethered 9-mer probes also showed good discrimination against mismatched DNA targets [23,24]. A particularly intense hybridization signal was obtained when a glass-tethered 9-mer probe hybridized contiguously with one auxiliary oligonucleotide pre-annealed to a specific target DNA sequence [30]. The increased hybrid

stability is apparently caused by strong base-stacking interactions between the capture probe and the auxiliary oligonucleotide. The use of auxiliary oligonucleotides pre-annealed to single-stranded (ss) DNA targets provides a convenient means for labelling these DNA molecules, disrupting secondary structures in the region of analysis, covering any redundant binding sites in the DNA targets, and inducing tandem hybridization (contiguous base stacking) with the capture probes to achieve efficient mismatch discrimination particularly at the terminal site of the DNA probes [31].

A simple procedure for the covalent attachment of oligonucleotide probes to glass surfaces has recently been discovered [32]. The method consists of coupling directly 3'-propanolamine-derivatized oligonucleotides to underivatized glass slides (see Fig. (2)). The glass-oligonucleotide linkage demonstrated stability in hot water and, thus, enabled multiple cycles of hybridization. The linkage was also stable

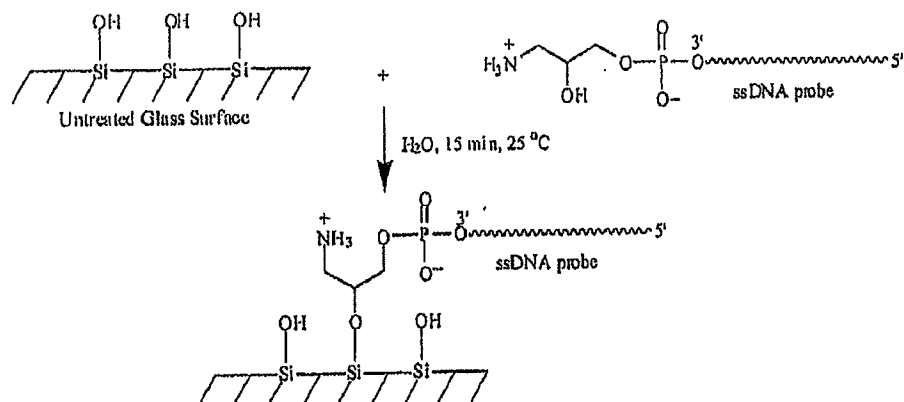


Fig. (2). Direct attachment of 3'-aminated oligonucleotides to underivatized glass slides.

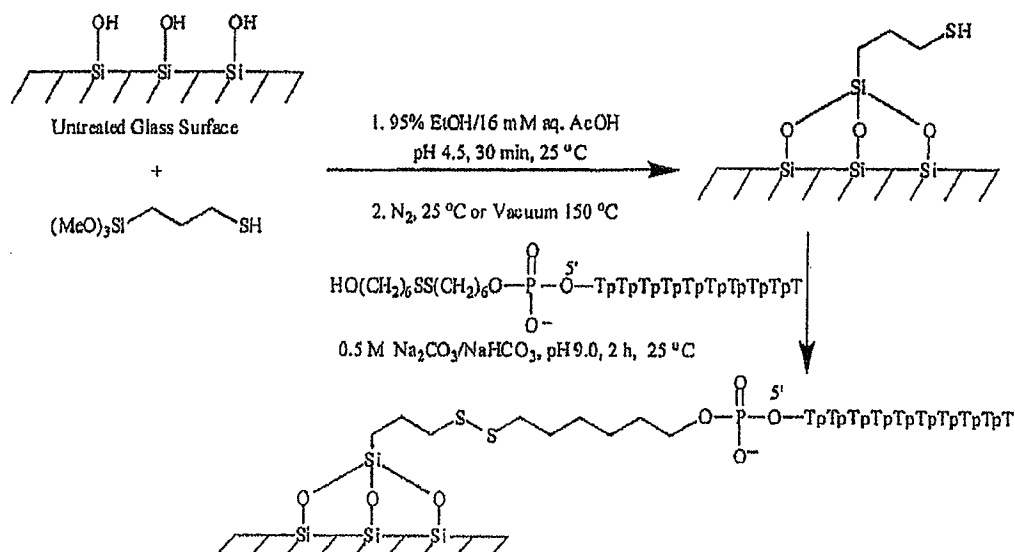


Fig. (3). Covalent attachment of 5'-disulfide-modified oligonucleotides to glass surfaces via a thiol/disulfide exchange reaction.

to mild acids but unstable to mild bases. Interestingly, the glass-oligonucleotide linkage was not formed with oligonucleotides functionalized with a hexylamine linker at the 5'-terminus. In addition, linkage formation was inhibited by treatment of the glass surface with propanolamine but not with propylamine. Furthermore, linkage formation did not occur upon acetylation of the primary amine group of the propanolamine-functionalized oligonucleotides, and thus indicated that linkage formation was assisted by the amine group. The attachment reaction proceeded rapidly in aqueous solution at ambient temperature and gave a lower background of non-specific DNA target binding to the surface when compared with that obtained with epoxysilanized glass under the same conditions. The attachment density was 10^{10} - 10^{11} oligonucleotides/mm² [32].

Another approach to the tethering of oligonucleotides to glass slides involved the functionalization of oligonucleotides at the 5'-terminus with activated 1-*O*-dimethoxytrityl hexyl disulfide 1'-[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite [33]. Glass slides were treated with 25% ammonium hydroxide and, then, immersed in a solution of 1% 3-mercaptopropyl trimethoxysilane, 95% ethanol, and 16 mM acetic acid (pH 4.5) (see Fig. (3)). Glass slides were cured under vacuum for 2 h at 150 °C or overnight under nitrogen at ambient temperature. Attachment of 5'-disulfide-modified oligonucleotides to the glass surfaces was effected according to a thiol/disulfide exchange reaction at pH 9 (Fig. (3)). The density of oligonucleotide attachment reached a plateau at an oligonucleotide concentration of ca. 20 μ M. A maximal attachment density of ca. 3.0×10^5 probes/ μ m² was measured. Given that no measurable non-specific attachment was detected, the accessibility of immobilized oligonucleotides for target template hybridization was preserved without additional blocking or stripping steps. ³²P-Labelled oligonucleotides were used to evaluate the accessibility and specificity of surface-bound DNA probes for hybridization. It was found that hybridization efficiency was directly related to the capture probe attachment density. Although disulfide bridges can be

cleaved with reducing agents such as dithiothreitol (DTT), these linkages were stable to hybridization conditions. No significant cleavage of the disulfide-linked oligonucleotides was observed when in contact with biological samples or with PCR and polymerase extension reaction mixes. The stability of printed arrays in storage is such that the arrays remained functional for at least one month when stored under dry and cold (4 °C) conditions. Storage stability can be extended as long as the arrays are not exposed to high humidity, a reducing reagent, or extreme high- or low-pH conditions [33]. This oligonucleotide immobilization chemistry provides an efficient and inexpensive method for both research and large-scale DNA array preparations. DNA oligonucleotide arrays generated from 5'-disulfide oligonucleotides have been used for probing the sequence of a 33 base region of exon 8 of the p53 tumor suppressor gene for missense, insertion, and deletion mutations [33].

Untreated glass slides can also be silanized by immersion in a solution of 2% *p*-aminophenyltrimethoxysilane in acetone:water (1:1 v/v) or in a solution of 2% 3-aminopropyltriethoxysilane in acetone [34] (Fig. (4)). Synthetic oligonucleotides were aminoalkylated at the 5'-terminus upon reaction with a commercially available 5'-aminomodifier C6 phosphoramidite derivative. The 5'-aminoalkylated oligonucleotides were then treated with 0.1 M succinic anhydride in dry DMF. After deprotection and purification, the succinylated DNA oligonucleotides were spotted on aminosilanized glass slides in the presence of aqueous 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. The efficiency of oligonucleotide attachment was determined by the use of a 5'-succinylated oligonucleotide labelled at the 3'-end by the addition of [α -³²P]ddATP catalyzed by terminal transferase. It was demonstrated that the coupling of oligonucleotides to the glass surface was 3- to 10-fold more efficient at pH 3.6 than at pH 6.8. Under these conditions, 39-50% of the 10^9 to 10^{12} DNA oligonucleotide molecules that were deposited became covalently attached to the glass surface. ³²P-Labelled DNA target oligonucleotides were used in the evaluation of

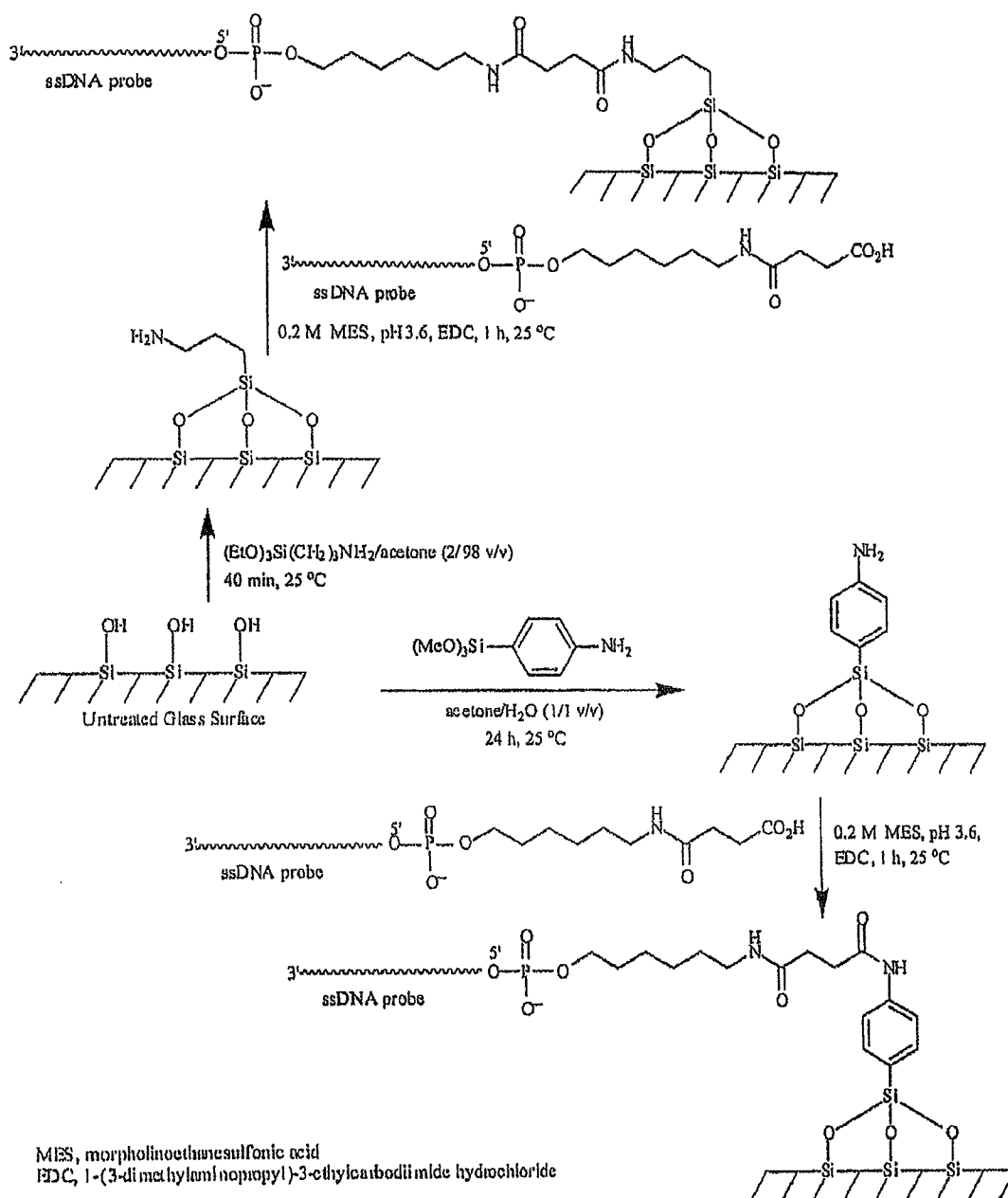


Fig. (4). Covalent attachment of 5'-carboxyalkylated oligonucleotides to aminosilanized glass surfaces.

hybridization efficiencies. It was argued that when an excess of surface-bound oligonucleotides was present, 26-74% of the DNA targets were captured by hybridization. More DNA targets hybridized to surface-bound oligonucleotides that had a 15 nt spacer than to those immobilized oligonucleotides without a spacer sequence. When an excess of DNA targets was used, up to 90% of the surface-bound oligonucleotides were accessible for hybridization [34]. Non-specific binding of unrelated ^{32}P -labelled DNA oligonucleotide targets was less than 0.05%.

Glass slides treated with aminopropyltrimethoxysilane can alternatively be reacted with excess 1,4-(phenylene) diisothiocyanate to generate activated glass surfaces that can

be stored indefinitely at 4 °C in a vacuum desiccator without apparent loss of reactivity [35]. 5'-Aminoalkylated oligonucleotides have been prepared by coupling *N*-trifluoroacetyl-6-aminohexyl-2-cyanoethyl *N',N'*-diisopropylphosphoramidite to the 5'-terminus of synthetic oligonucleotides according to solid-phase techniques. The purified 5'-aminoalkylated oligonucleotides were applied to a phenylisothiocyanate-activated glass slide in a sodium carbonate/bicarbonate buffer at pH 9.0 and incubated at 37 °C. The surface density of the covalently linked oligonucleotide probes was determined by the attachment of a 5'-aminoalkylated oligonucleotide ^{32}P -labelled at the 3'-terminus, and was found to be *ca.* 1250 Å²/oligonucleotide [35]. It has also been argued that the length of the spacer

between the glass surface and the DNA probe is important. For example, the oligonucleotide attachment chemistry presented in Fig. (5) provides a 23 atom linker between the surface and the DNA oligonucleotide. The length of such a linker was not sufficient for adequate hybridization. Upon addition of six to fifteen nucleotide spacers, hybridization signals became considerably stronger [35]. This system led to the direct fluorescence analysis of genetic polymorphisms. Five single-base mutations contained within exon 4 of the human tyrosinase gene were studied. By limiting the hybridization sequence length to 15 nt and keeping the GC content between 5 and 7 nt (out of the total 15 nt), a single set of hybridization and washing conditions provided all-or-none discrimination between perfect matches and single-base mismatches [35]. It should be noted that 4-nitrophenylchloroformate has also been used to activate aminopropylated glass surfaces. As illustrated in Fig. (5), the reaction of 5'-aminoalkylated oligonucleotides with these activated surfaces resulted in their covalent immobilization. It has additionally been demonstrated that the amino

function of DNA nucleobases can competitively react with the activated glass surfaces and interfere with subsequent hybridization reactions [36].

Immobilization of DNA oligonucleotides on fused silica (quartz) slides has been investigated [37]. The use of fused silica for DNA oligonucleotide attachment allows direct observation of the UV absorbance characteristic of DNA ($\lambda_{\text{max}} = 260 \text{ nm}$). Silanization of acid-cleaned quartz slides was performed using trimethoxysilylpropyldiethylenetriamine (PDETA), *N*-(2-aminoethyl)-3-aminopropyl trimethoxysilane (PEDA), or (3-aminoethyl-4-aminomethyl)phenetyl trimethoxysilane (EBEDA). Typically, PDETA-silanized quartz slides were immediately treated with the bifunctional crosslinker succinimidyl 4-[maleimidophenyl]butyrate (SMPB) to enable attachment of synthetic oligonucleotides that have in turn been prepared from thiol modifier C3-S-S-CPG columns (Fig. (6)). These columns led to the incorporation of a protected disulfide linkage into DNA oligonucleotides at the 3'-terminus.

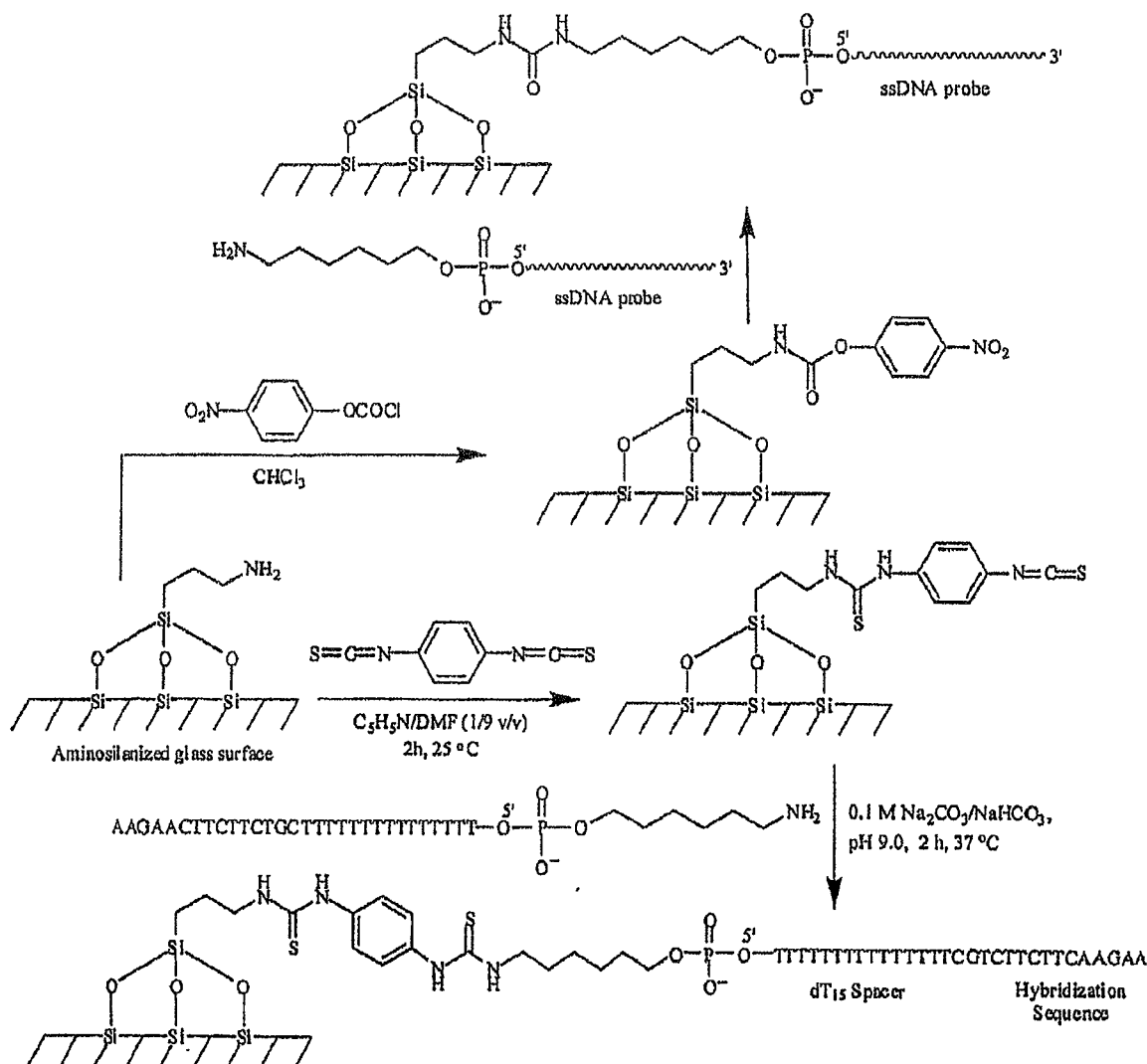


Fig. (5). Attachment of 5'-aminoalkylated oligonucleotides to aminosilanized glass surfaces activated with 1,4-phenylene diisothiocyanate or 4-nitrophenyl chloroformate.

Deprotected and purified oligonucleotides were treated with DTT to generate 3'-thiolated DNA oligonucleotides which were then coupled to crosslinker-treated silanized quartz slides under an inert atmosphere to minimize oxidation of the 3'-thiolated DNA oligonucleotides (Fig. (6)).

Reproducible attachment of the DNA oligonucleotides to the quartz surface was achieved with 1 μ M DNA solutions. The fused silica slides were incubated in a high salt buffer to remove non-covalently bound DNA oligonucleotides. According to UV spectroscopy, the A_{260} values obtained immediately after the DNA immobilization step fell by 50-70% after high salt treatment.

The surface density of covalently bound DNA was determined by coupling 5'-[32 P]-labelled 3'-thiolated DNA oligonucleotides on SMPB/PDETA-modified quartz slides, and was found to be *ca.* 20 pmol/cm² after treatment of the slides with a high salt buffer. When compared with other methods for DNA attachment to glass, this method appears

to be superior both in the density of immobilized DNA and the reduced concentration of DNA oligonucleotides (5-100 fold lower) required to achieve such an attachment density [37]. The ability of immobilized DNA oligonucleotides to form hybrids was much dependent on the sequence of the immobilized oligomers. Thus, 9 to 76% of the covalently immobilized oligonucleotides were amenable to hybrid formation. A prerequisite to the success of this oligonucleotide attachment chemistry is a rigorous control of the quality of the silanes used, and to expeditiously process the films from one step to the next to minimize side reactions. When used in conjunction with lithographic masks, this oligonucleotide attachment chemistry permitted the fabrication of patterned DNA surfaces which may be applied to the development of miniaturized DNA-based biosensors [38]. In this context, a similar oligonucleotide attachment chemistry has been used to covalently immobilize oligonucleotides on a spherical probe and a surface in an attempt to measure interaction forces between single strands of DNA with an atomic force microscope [39].

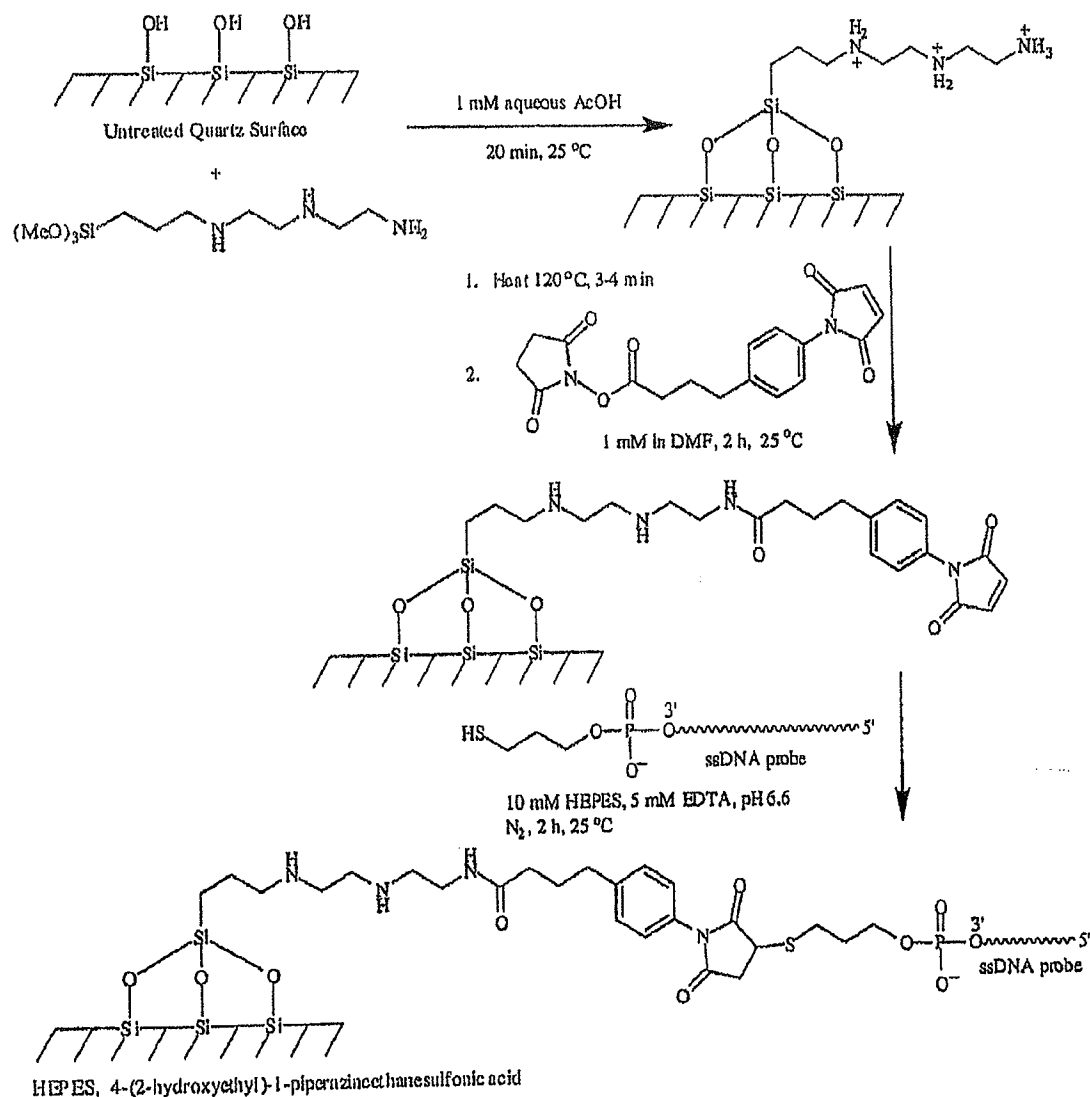


Fig. (6). Covalent attachment of 3'-mercaptoalkylated oligonucleotides to quartz surfaces functionalized with a spacer carrying a maleimido group.

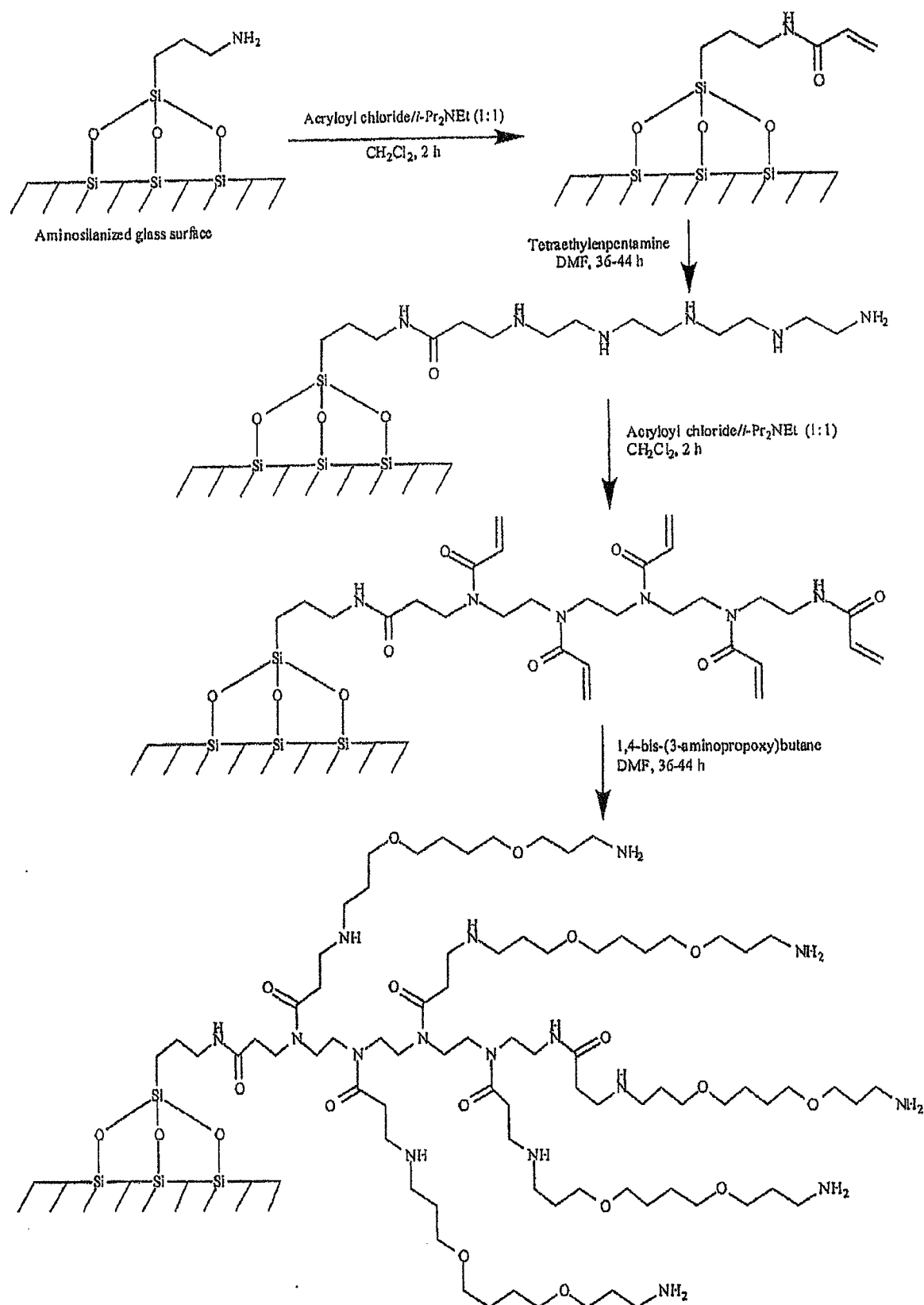


Fig. (7). Preparation of a dendrimeric linker structure on aminosilanized glass surfaces.

In an effort to improve the attachment of oligonucleotides to glass surfaces, a linker system has been devised. The process involved two chemical reactions that were successively performed and repeated until the desired linker type was produced. The first reaction was the acylation of a surface-bound amino group with either 4-nitrophenyl chloroformate or acryloyl chloride. The acylated surface was then reacted with diamines or polyamines to produce a linear or a branched, dendrimeric, linker structure [40]. The density of oligonucleotide attachment to the surface could therefore be increased in a very effective manner. As indicated in Fig. (7), acylation of an aminosilanized surface with acryloyl chloride followed by successive condensations with tetraethylenepentamine, acryloyl chloride, and 1,4-bis-(3-aminopropoxy)butane increased the number of reactive sites for the attachment of oligonucleotides by a factor of 10. Because polyamines contain both primary and slightly more nucleophilic secondary amino groups, not only one unique dendrimer (like the one shown in Fig. (7)) was generated but a mixture of dendrimers. To attach nucleic acid derivatives to the glass surface, a chemical linkage had to be formed between a functional group of these biomolecules and any of the amino groups of the dendrimeric linker. The amino functions of the glass surface were activated upon reaction with homofunctional crosslinking agents such as 1,4-phenylene diisothiocyanate (PDITC), disuccinimidyl carbonate (DSC) and dimethylsuberimide (DMS). The covalent attachment of 5'-aminolinked oligonucleotides, PNA oligomers, and amino-terminated PCR DNA fragments was best achieved when the aminated glass surface was activated with PDITC or DMS in agreement with the findings of Guo *et al.* [35]. Following oligonucleotide attachment, the glass surface was deactivated by treatment with 6-amino-hexanol to maintain surface hydrophilicity. This deactivating step quantitatively blocked all remaining reactive functionalities on the surface and prevented the non-specific binding of DNA targets that can cause high background [40]. Only 5'-amino-functionalized oligonucleotides were found covalently attached to the glass surface after more than two hybridization and stripping events. Oligomers attached through an hydroxyl function were not stable to repeated hybridization and stripping steps, as they vanished from the glass surface. Only little covalent immobilization occurred, if any, via the amino function of DNA nucleobases. In this regard, because of the 5'-terminal attachment of oligonucleotides to the glass, the entire sequence of each oligonucleotide was accessible for hybridization with DNA targets. Surface attachment of peptide nucleic acid (PNA) oligomers gave increased signal intensities relative to those obtained with oligonucleotides because of higher PNA binding affinity in hybridization reactions.

It should be noted that the dendrimeric structure of the linker did not affect the kinetics of hybridization to either oligonucleotides or PCR DNA products [40]. DNA arrays that have been prepared according to this method are reusable; these arrays withstood more than seven consecutive cycles of hybridization and stripping without any significant loss of signal intensity on imaging.

The use of a two-dimensional optical wave guide and light scattering labels to detect hybridization of DNA targets

on oligonucleotide arrays is a rapid and cost-effective method for distinguishing sequence variations from known sequences [41]. Basically, the evanescent wave created by illumination of a wave guide glass slide is used to scatter light from particulate labels adsorbed on the DNA capture sites of the wave guide surface. Given that an evanescent wave only extends 100-300 nm from the wave guide surface, unbound or dissociated labels do not scatter light and only those labels bound to the surface generate signals. DNA chips for wave guide detection were constructed by immobilizing pre-synthesized 3'-aminated oligonucleotides in 0.5 μ L spots on glass slides coated with casein (Fig. (8)). Solutions of 3'-biotinylated ssDNA targets in a Tris buffer at pH 7.8 were applied to the surface of the wave guide and allowed to stand for 5 min at 25 °C. After draining off the excess 3'-biotinylated DNA, a solution of anti-biotin antibody conjugated to selenium particles was spread over the surface and left standing for 1-5 min. The DNA chips were washed and then stored in phosphate-buffered saline (PBS) before imaging under wave guide illumination. Light from a 150 W lamp was supplied through one edge of the wave guide slide by the use of a slit. Light scattering from the selenium conjugates adsorbed on the wave guide chips was observed visually or recorded using a charge-coupled device (CCD) camera for quantitative measurements [41]. Dilution experiments revealed an apparent lower limit of detection at 0.4 nM oligonucleotides. Temperature-controlled wave guide chips allowed the elaboration of thermal DNA denaturation (melting) curves that were consistent with those obtained in liquid phase, and permitted the facile detection of single base-pair mismatches. The system also permitted real-time binding or melting of a light-scattering label on the array. This methodology should be particularly useful for diagnostic applications and in the generation of nucleic acid sequence information via sequencing by hybridization (*vide infra*).

Avidin also binds to glass surfaces by physical adsorption. The physically adsorbed avidin can be further stabilized by treatment with glutaraldehyde; this reagent effects the formation of crosslinks between the protein and the glass surface. The avidin-conjugated glass surface can then tightly bind to, for example, a biotinylated single-stranded DNA "molecular beacon" to generate a DNA-covered glass surface. Molecular beacons [42] are single-stranded oligonucleotide probes that can fold into stem-and-loop structures. The loop portion of a beacon is complementary to specific nucleic acids. A sequence of five nucleotides at each terminus of a molecular beacon is complementary to each other and forms a stem. A fluorophore (F) is linked to one end of the beacon, whereas a fluorescence quencher (Q) is attached to the other end as depicted in Fig. (9). The stem keeps F and Q near each other causing the fluorescence of F to be quenched by energy transfer. However, when the molecular beacon probe encounters a complementary DNA target, a hybrid that is longer and more stable than the stem is formed. Spontaneous conformational reorganization forces the stem to dissociate. As a consequence, the fluorophore F moves away from the quencher Q and restores fluorescence. Thus, molecular beacons emit an intense fluorescent signal at room temperature only when hybridized to complementary nucleic acid targets. These DNA molecules exhibit high selectivity for single base-pair mismatch identification and

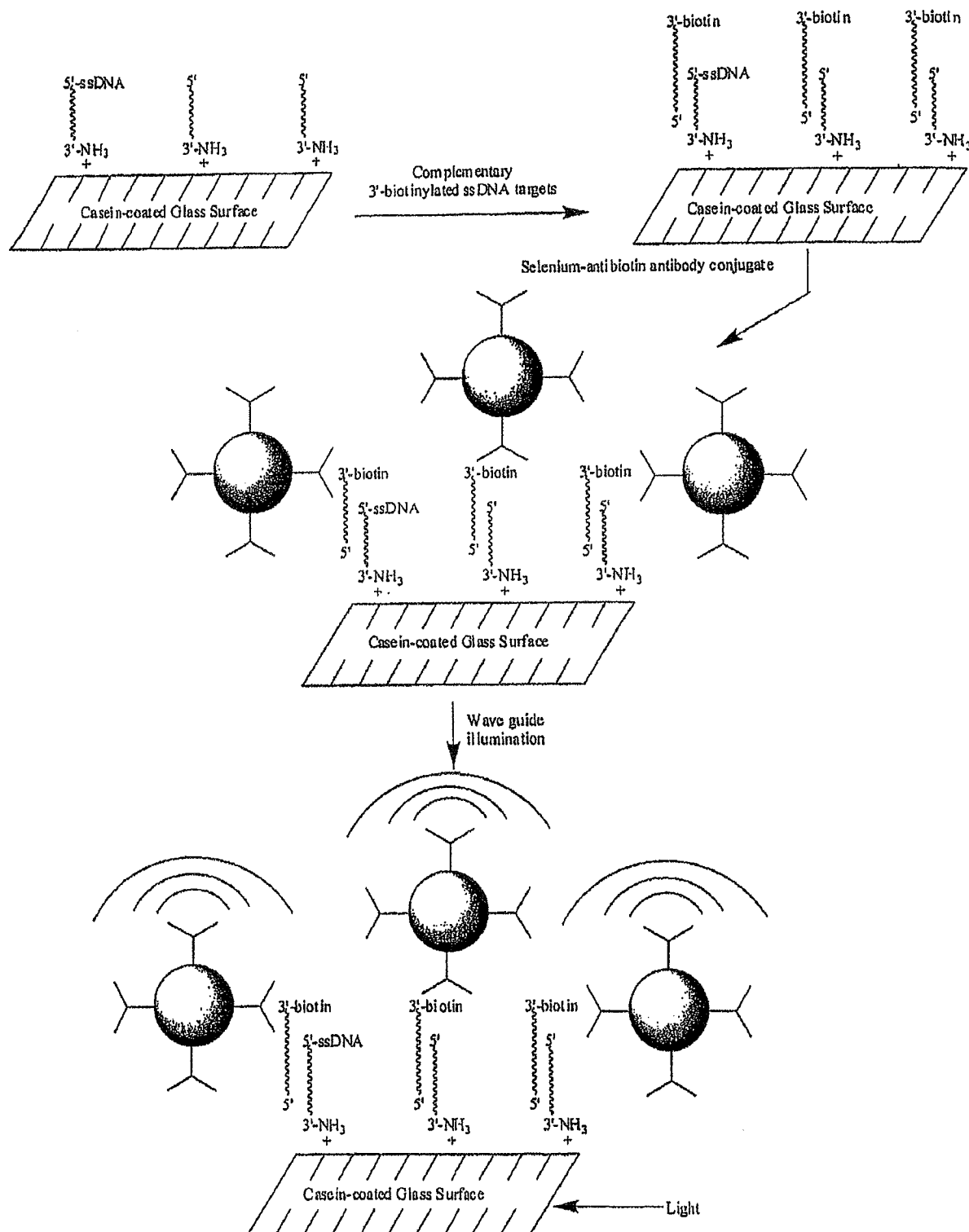


Fig. (8). DNA hybridization-induced light scattering of selenium conjugates adsorbed on wave guide glass slides.

show great promise as diagnostic tools in the detection of genetic diseases. A biotinylated single-stranded DNA molecular beacon has been synthesized from DABCYL-derived CPG (controlled-pore glass) [43]; DABCYL [(4-dimethylaminophenylazo) benzoic acid] is a non-fluorescent

chromophore acting as a general fluorescence quencher Q. Incorporation of a T phosphoramidite derivative biotinylated at C-5 into the DNA chain near Q within the five base-pair stem was performed according to standard solid-phase protocols. The DNA chain was extended to include the loop

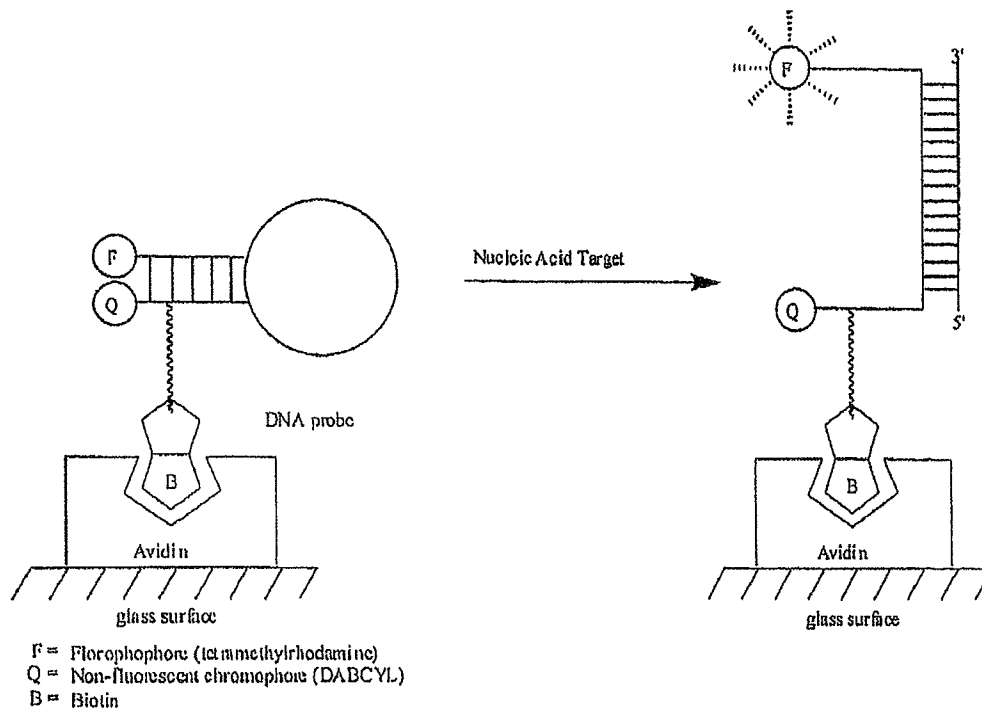


Fig. (9). Immobilization of a biotinylated molecular beacon on avidin-coated glass surfaces.

of the molecular beacon (18 nt) and terminated by the addition of a 5'-aminoalkylated phosphoramidite derivative to permit conjugation with tetramethylrhodamine. The biotinylated DNA molecular beacon was purified by gel filtration and RP-HPLC chromatography, and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). The beacon was then immobilized on the avidin-coated glass surface; the binding process was fast and efficient. The immobilized DNA molecular beacon stayed with the surface even after immersion in buffered solutions for a few days. Hybridization of the molecular beacon with its complementary DNA target occurred at DNA concentrations ranging from 5 to 600 nM. Fluorescence intensity measurements indicated that immobilized DNA molecular beacons can be used to detect DNA molecules in the subnanomolar range with high specificity [43]. It should also be pointed out that PNA-DNA molecular beacons have been immobilized on streptavidin-coated microtitre plates to enable rapid detection of PCR amplicons [44].

The next section will address the attachment of pre-synthesized oligonucleotides on glass surfaces covered with a thin film of gold.

IMMOBILIZATION OF OLIGONUCLEOTIDES ON GOLD SURFACES

A new method for the preparation of oligonucleotide arrays on gold surfaces has been reduced to practice. This method relies on an *in situ* surface plasmon resonance (SPR) imaging detection system to study DNA hybridization [45]. A surface plasmon is an electromagnetic wave propagating along the interface between a metal and a dielectric [46]. The

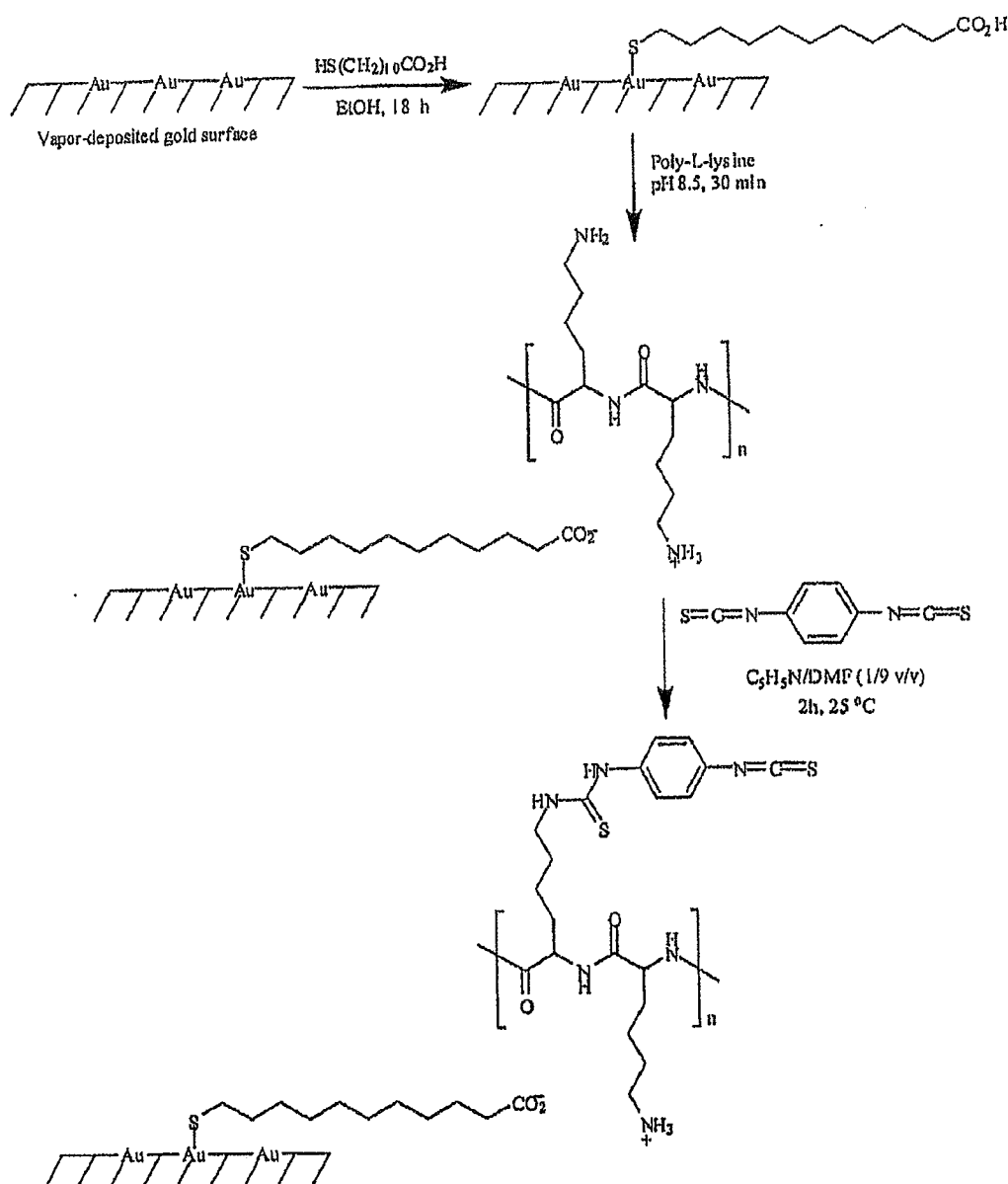
detection technique is able to differentiate between single- and double-stranded DNA regions on the gold surface without labelling DNA. This property is an advantage over most DNA hybridization detection systems, as it simplifies DNA sample preparation and eliminates any possible perturbation caused by the label on normal DNA interactions. Because the SPR technique allows one to observe changes occurring at the surface in real time, there is no need to wash excess DNA analyte. This method permits the *in situ* monitoring of hybridization and denaturation as they happen. This feature is desirable because in most DNA hybridization techniques it is difficult to determine how much washing is necessary to achieve mismatch discrimination. The SPR technique is also suitable for thermodynamic studies of DNA hybridization given that it can detect surface-bound dsDNA in equilibrium with ssDNA in solution above the surface. SPR occurs only at specific metallic surfaces. Thin gold films were selected for the construction of oligonucleotide arrays. Typically, glass microscope slides are rigorously cleaned and, then, silanized with (3-mercaptopropyl)trimethoxysilane before gold can be vapor-deposited to a film thickness of 47 nm [47]. The gold surface is further treated with 11-mercaptoundecanoic acid to covalently attach carboxylic groups to the surface (Fig. (10)). A layer of poly-L-lysine is then electrostatically adsorbed onto the carboxylated surface. The aminated layer is reacted with 1,4-phenylene diisothiocyanate to permit the coupling of 5'-aminoalkylated oligonucleotides to the resulting isothiocyanate-derivatized surface in a 1.5 μ L droplet of ca. 2.0 mm diameter (Fig. (10)). Hybridization of both oligonucleotides and strand-separated PCR DNA fragments was detectable. However, a slower hybridization kinetics for the PCR fragments was observed. Temperature-controlled SPR experiments allowed the discrimination of perfectly

matched duplexes and single base-pair mismatched duplexes. Even though SPR detection does not require DNA labelling, fluorescently-labelled DNA targets were employed to verify and confirm the hybridization to verify and confirm the behavior of these DNA arrays by fluorescence imaging. Thus, the *in situ* SPR imaging method for the detection of DNA hybridization shall complement other existing methods for studying DNA interactions and might find applications in mutation screening assays.

It must, however, be cautioned that poly-L-lysine electrostatically adsorbed onto gold surfaces will desorb on exposure to either high or low pH solutions [47]. This feature may nonetheless be useful in the construction of those biosensors that need to be regenerated. It should also be noted that the covalent attachment of poly-L-lysine to carboxylated gold surfaces has been reported [48]. Treatment of a carboxylated gold film with 1-(3-dimethylaminopropyl)-

3-ethylcarbodiimide hydro-chloride (EDC) and *N*-hydroxysulfosuccinimide (NHSS) resulted in the formation of active esters on the gold surface. As profiled in Fig. (11), the reaction of poly-L-lysine with these active esters led to the formation of covalent amide linkages. Further derivatization of the covalently attached poly-L-lysine with sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate afforded a gold surface functionalized with maleimide groups to enable subsequent attachment of sulfhydryl-tagged oligonucleotides [48].

Another strategy for the covalent immobilization of single-stranded oligonucleotides on gold-covered planar surfaces has been described [49]. The strategy consisted of the stepwise formation of two covalently linked monolayers to provide a stable structure with defined molecular density and spatial orientation in each layer. As illustrated in Fig. (12), the gold surface was incubated in an ethanol solution of



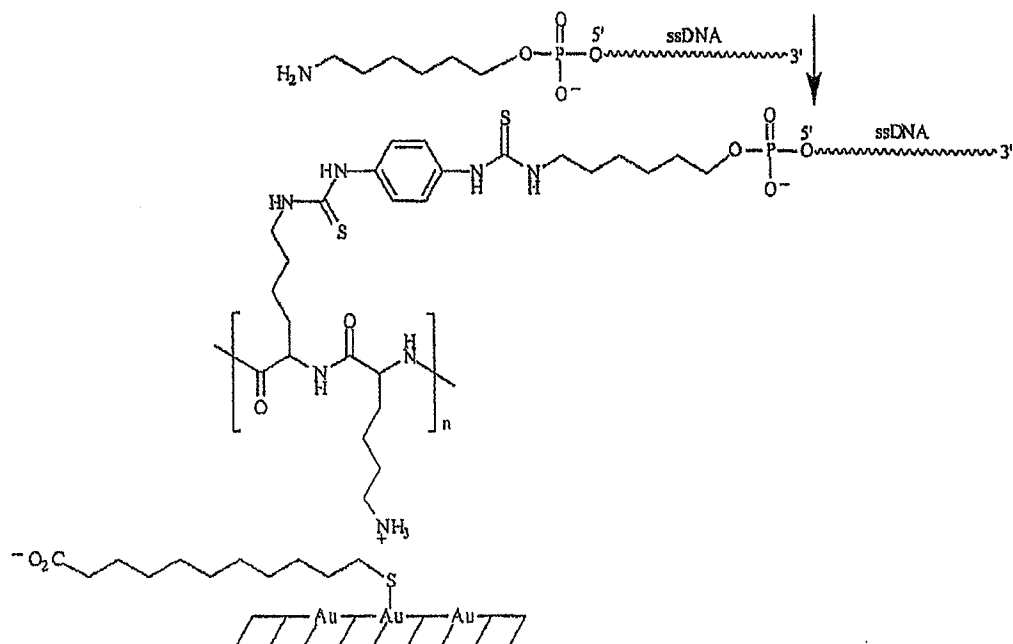


Fig. (10). Immobilization of oligonucleotides on vapor-deposited gold films.

1-mercaptoundecanoic acid (0.2-2 mol%) and 1-decanethiol for 12 hr. The carboxylated gold surface was then activated by treatment with *O*-(*N*-succinimidiyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate and *N,N*-

diisopropylethylamine in acetonitrile. The conjugation of 5'-aminoalkylated oligonucleotides to the activated ester groups of the gold surface was performed in DMF containing 2% deionized water and 1% *N,N*-diisopropylethylamine [49].

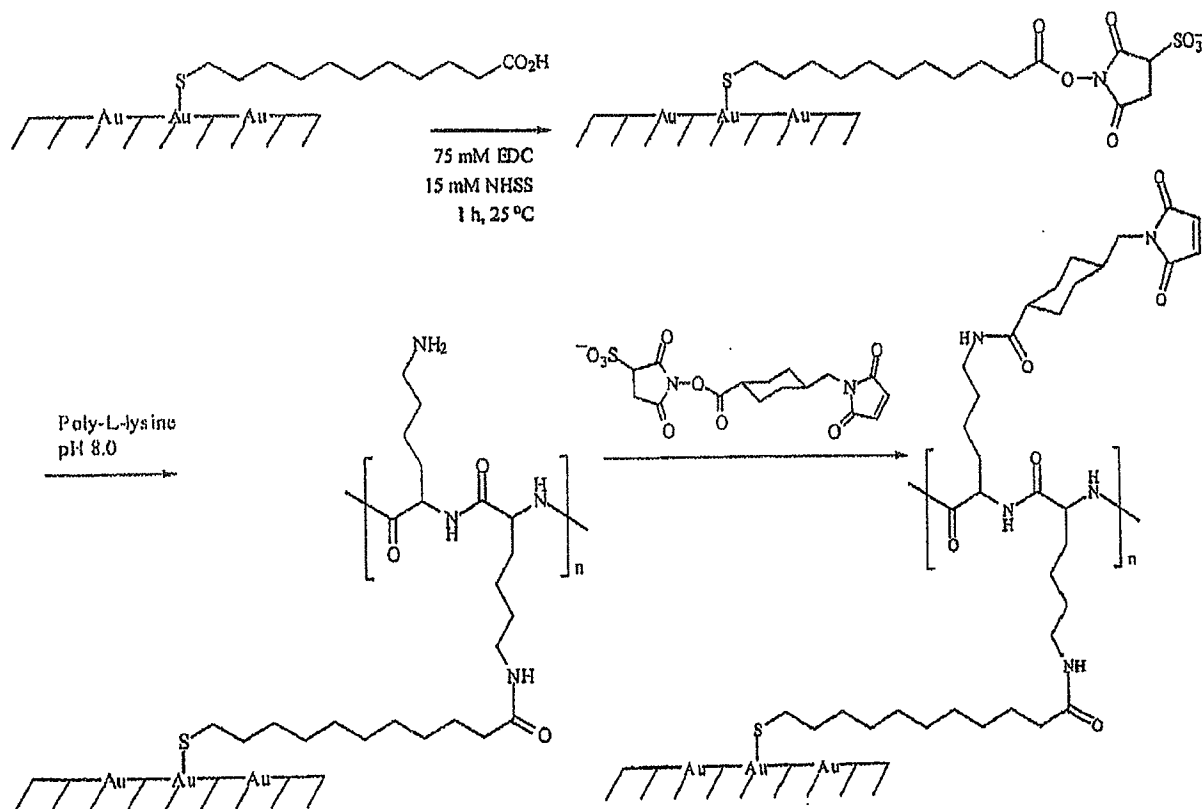


Fig. (11). Covalent attachment of poly-L-lysine to gold films and its functionalization for immobilizing mercaptoalkylated oligonucleotides.

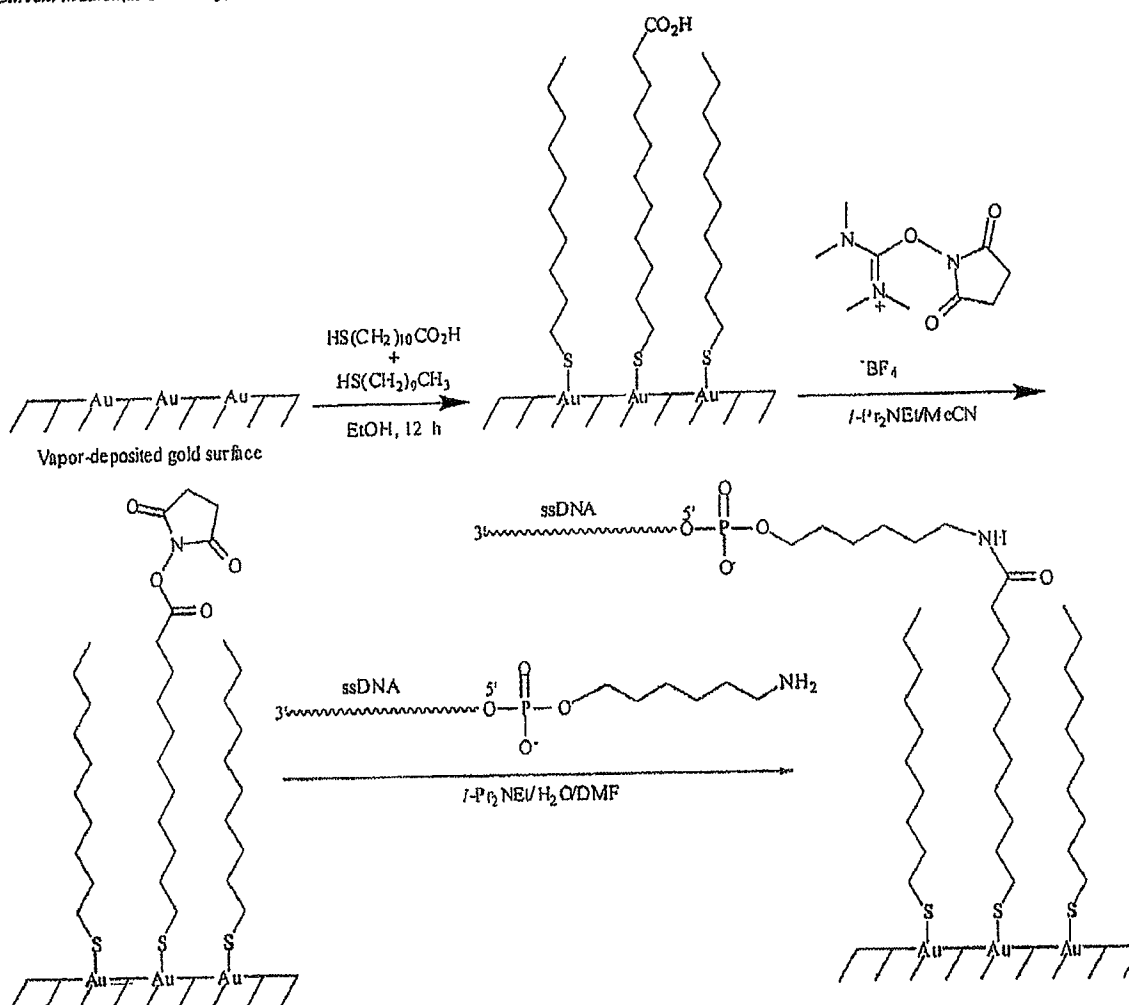


Fig. (12). Covalent attachment of 5'-aminoalkylated oligonucleotides on gold surfaces derivatized with activated ester functions.

The surface density of the oligonucleotides can be controlled by selecting the proper concentration of 1-decanethiol. Furthermore, the thioalkyl layer prevented non-specific oligonucleotide-gold interactions from occurring especially when oligonucleotides are thiol-derivatized prior to immobilization [50,51]. A maximum of $ca. 3 \times 10^{12}$ oligonucleotides/ cm^2 was obtained and an excellent hybridization efficiency ($ca. 90\%$) was achieved. Surface plasmon resonance was used to measure the surface density of immobilized and hybridized oligonucleotides. Additionally, real-time identification of oligonucleotides during immobilization and hybridization in an aqueous environment was achieved for the first time using attenuated total internal reflection (ATR) Fourier transform infrared (FTIR) spectroscopy [49]. Thus, well-defined and covalently immobilized oligonucleotides on gold surfaces may find application in the development of novel DNA-based sensors for genetic analysis and the study of nucleic acid-ligand interactions.

A selective colorimetric detection of a polynucleotide target using gold nanoparticle probes has recently been reported [52]. Gold nanoparticles ($ca. 13 \text{ nm}$ diameter) conjugated to 3'- and 5'-mercaptoalkylated oligonucleotide

probes led to the formation of a complex with a 24-base polynucleotide target in a "tail-to-tail" arrangement. The nucleic acid complex triggered a red to purple color change in solution. The color change is due to a red shift in the surface plasmon resonance of the gold nanoparticles [52]. The complex exhibited sharp "melting transitions" allowing one to distinguish target sequences containing one base-end mismatches, deletions, or an insertion from the fully complementary target [52]. This new one-pot colorimetric detection method may prove useful in diagnosing genetic diseases that are characterized at the DNA level by single nucleotide mutations.

Synthetic oligonucleotides can also be covalently attached to polyacrylamide gel pads constructed on glass surfaces. The details of this technology will be reviewed in the following section.

GEL-IMMOBILIZED OLIGONUCLEOTIDES ON GLASS SURFACES

It has been demonstrated that a glass plate covered with a $30 \mu\text{m}$ -thick layer of polyacrylamide provides a suitable

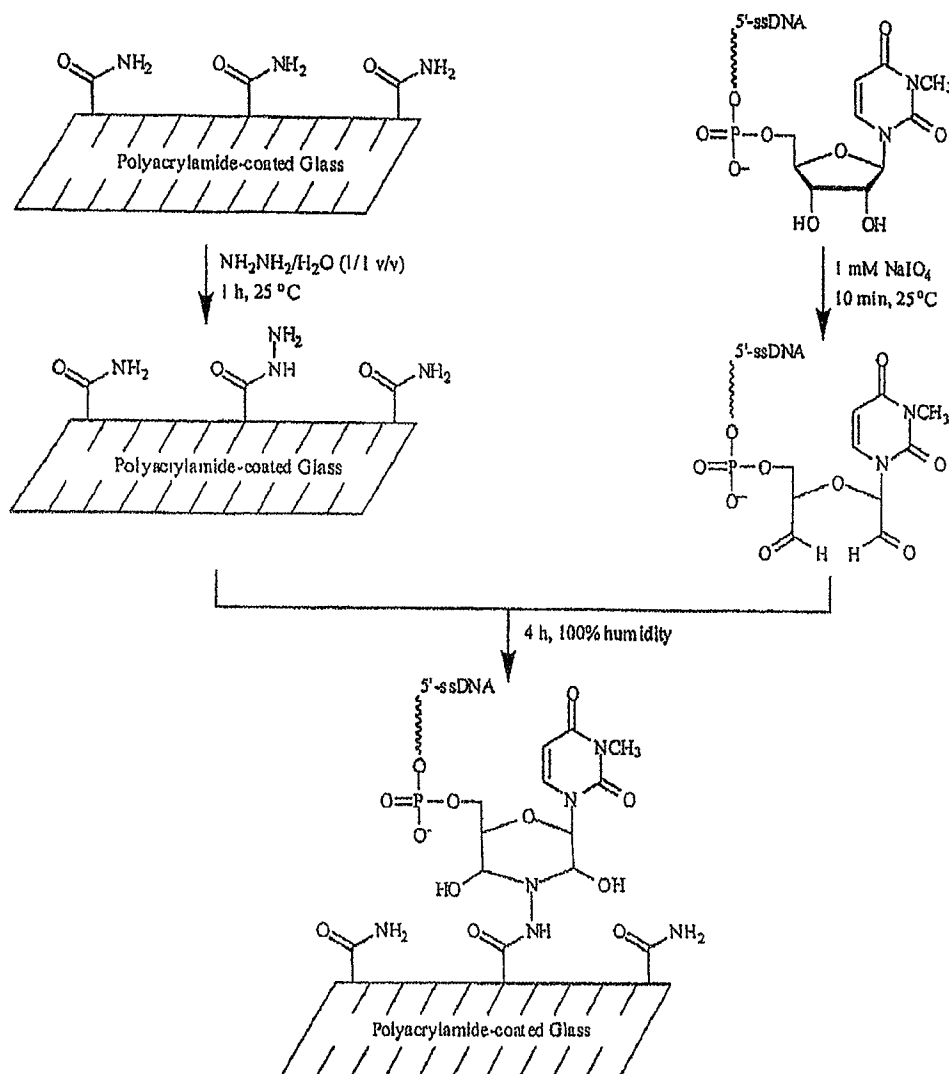


Fig. (13). Covalent attachment of 3'-modified oligonucleotides to polyacrylamide-coated glass surfaces.

immobilization support for oligonucleotides [53,54]. The gel was activated by converting a number of amide groups to hydrazides by treatment with aqueous hydrazine. Oligonucleotides (8-mers) with 3'-terminal N^3 -methyl uridine were mixed with sodium periodate to generate terminal aldehyde groups. Oxidized oligonucleotide solutions were then pipetted onto an activated gel plate to provide an array of 1 mm wide dots of immobilized oligonucleotides (Fig. (13)). Experiments with periodate oxidized [α - ^{32}P]-UMP indicated that the capacity of the gel was *ca.* 1 nmol/mm². The attachment of oligonucleotides to the gel was stable enough to sustain at least five hybridization/wash cycles without noticeable loss of hybridization sensitivity [54]. The immobilized oligonucleotides were hybridized to either ^{32}P -labelled oligonucleotides (17-mers) or to oligonucleotides that have been labelled with tetramethylrhodamine at their 3'-termini. These hybridization experiments distinguished perfect duplexes from those containing mismatches. The detection limit of fluorescent dot hybridization on polyacrylamide-

coated glass plates was *ca.* 10 attomoles of DNA target per dot [54]. These data suggest that DNA chips constructed according to this technology can be used for DNA mapping and identification of DNA sequence polymorphisms. However, the hydrazide chemistry for oligonucleotide attachment to the gel may not provide sufficient stability for some applications.

Functionalized gels for oligonucleotide microchips should meet specific requirements such as: (i) easy preparation and modification of the gels; (ii) high and reproducible oligonucleotide immobilization yields; (iii) long-term storage stability of the activated gels; (iv) highly stable oligonucleotide attachment; and (v) low non-specific binding of nucleic acid targets. It is important to note that glass slides treated with 3-(triethoxysilylpropyl)acrylamide provided 20 μm polydimethylacrylamide films exhibiting considerably better resistance to chemical modifications. As outlined in Fig. (14), copolymerization of N,N -dimethylacrylamide and N -(5,6-di-*O*-isopropylidene)

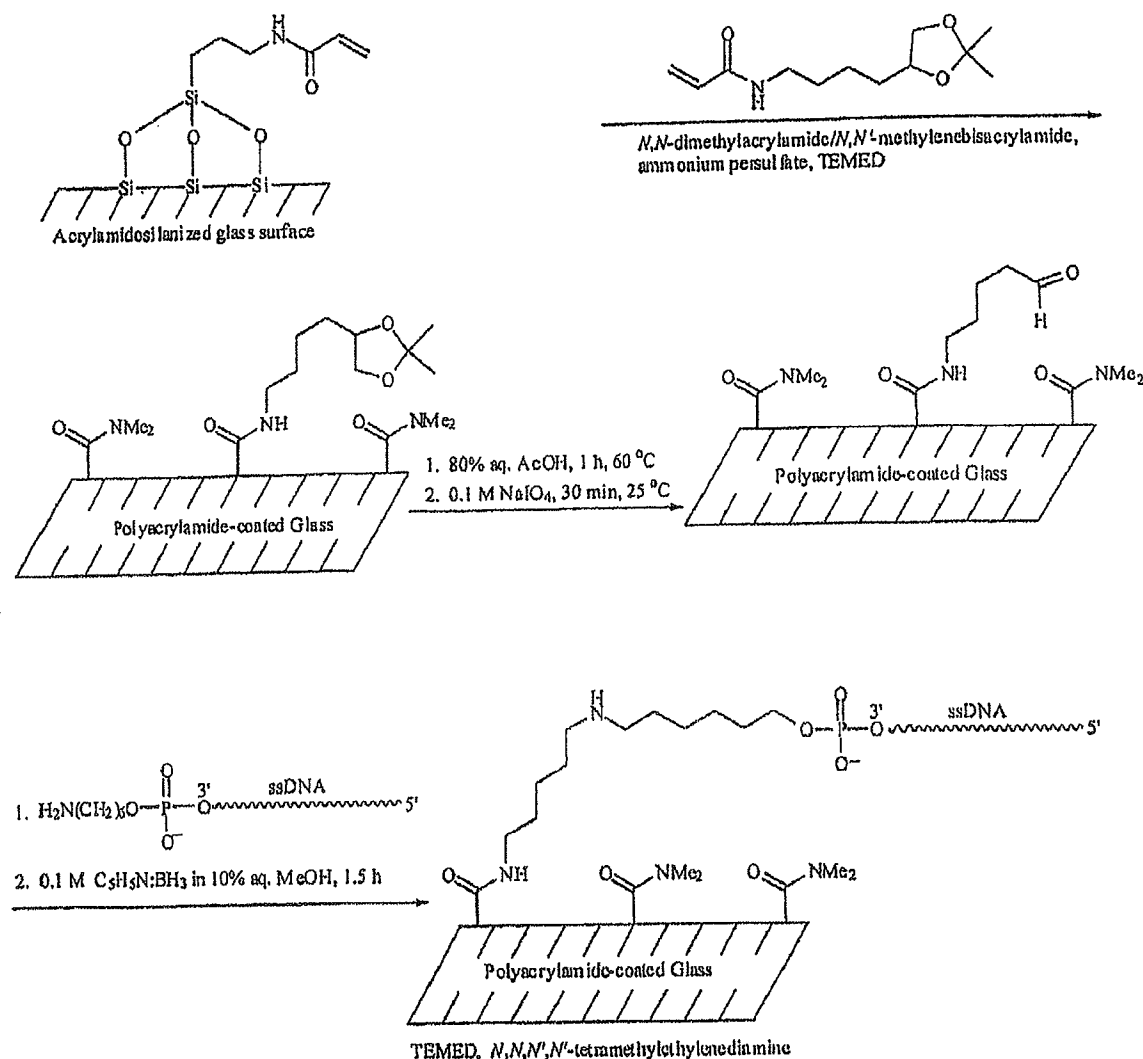


Fig. (14). Covalent immobilization of aminoalkylated oligonucleotides on polyacrylamide-coated glass surfaces.

hexylacrylamide in the presence of *N,N*-methylene-bis-acrylamide and ammonium persulfate ultimately resulted in a gel functionalized with aldehyde groups. In order to determine the efficiency of oligonucleotide immobilization on an aldehyde matrix, 5'-[³²P]-labelled 3'-aminoalkylated oligonucleotides (8-mers) were applied to the gel surface and mixed with a reducing reagent such as sodium cyanoborohydride or the pyridine-borane complex [55]. After exhaustive washing of unbound oligonucleotides, each spot (*ca.* 1 mm diameter) contained 200-500 pmoles of immobilized DNA oligonucleotides.

Aldehyde gels led to efficient oligonucleotide attachment but produced higher levels of non-specific binding (8%) of control octamers relative to that measured on gels using an hydrazide attachment chemistry (0%) [54]. The amino function of DNA nucleobases may presumably exhibit some reactivity toward the aldehyde groups of the matrix and contribute to non-specific binding. The stability of oligonucleotide attachment points was considerably greater on aldehyde matrices after reduction (*t*_{1/2} = 250-500 h) than

on hydrazide gels (*t*_{1/2} = 33-50 h) as measured by residual radioactivity on each gel type after extensive washing with a pH 7.0 buffer at 60 °C. It is interesting to note that regardless of the type of oligonucleotide attachment, the two gel types showed a rapid loss of the bound material (*ca.* 20%) within the first 2 h. Further loss of bound material was slower by factors of 10-100. The relatively rapid initial breakdown of the gel matrix appears to be caused by the elimination of short oligonucleotide-containing polyacrylamide or polydimethylacrylamide chains that were not linked to the entire polymer.

Thus, from all the methods that have been tested [55], high-yield immobilization of 3'-aminoalkylated oligonucleotides on aldehyde gels resulted in stable attachment, and may be viewed as the most efficient procedure for oligonucleotide gel-microchip preparation. Furthermore, this technology unlike the hydrazide attachment chemistry does not require additional steps for the oxidation of terminal 3'-ribonucleosides, and permits

oligonucleotide immobilization *via* either the 3'- or 5'-terminus.

Over the years, a number of methods have been described for the attachment of oligonucleotides to polyacrylamide gels [56] amenable to the construction of DNA chips. A three-dimensional polyacrylamide gel provides more than 100-fold greater capacity (nmol/cm^2) for immobilization than does a two dimensional glass support [57,58]. More than 70% of the well-spaced oligonucleotides can hybridize with fluorescently-labelled DNA targets. This three-dimensional matrix prevents interference between different oligonucleotides and DNA targets during hybridization, and enhances the efficiency of discrimination of perfect duplexes from mismatched ones [58]. A chip that is $1 \times 1 \text{ cm}$ may contain 20,000-30,000 gel elements that are $40 \times 40 \mu\text{m}$ each.

Recently, oligonucleotides derivatized at the 3'-terminus with an allyl phosphodiester function [such as $d(\text{Ap})_{10}\text{-allyl}$] were copolymerized with acrylamide-bisacrylamide in the presence of ammonium persulfate and N,N,N',N' -tetramethylethylenediamine (TEMED) onto the surface of a glass slide pre-treated with 3-(triethoxysilyl)propylacrylamide [59]. Despite the lower activity of allyl groups in copolymerization compared with acrylamide, the level of incorporation can be increased considerably by introducing several allyl groups into any oligonucleotide [59]. Fluorescently-labelled complementary oligonucleotides [such as Texas Red- pdT_{10}] were hybridized to the gel matrix resulting in the capture of the fluorescent target in the gel pads; no fluorescence was observed in those gel elements that did not contain polymerized allyl-oligonucleotides [59].

A limitation of gel-based DNA chips is the size of the DNA targets that can diffuse into the gel. The use of short DNA targets eliminates such a limitation, and decreases the interference of hairpin structures on hybridization with immobilized oligonucleotides [58].

The wide range of oligonucleotide immobilization density on gel pads increases sensitivity in the detection of fluorescently-labelled DNA targets, allows the determination of thermodynamic parameters for perfect and mismatched DNA duplexes [60,61], and permits contiguous stacking hybridization to increase the sequencing efficiency of oligonucleotide microchips [62] (*vide infra*). In this regard, when DNA targets are hybridized to octanucleotides immobilized on a gel-based DNA chip, the presence of pentanucleotides produced rather stable 13 bp duplexes; a mismatch in the pentanucleotide part of the duplex prevented its formation. Such a strategy can increase the length of the DNA to be sequenced from 200 up to 4,000 bases [58]. These microchips have also been applied to the sequence analysis of nucleic acids for identification of genetic mutations. For example, β -thalassemia mutations were detected in patients upon hybridization of PCR-amplified DNA with gel pad oligonucleotide microchips [58].

Both single- and double-stranded (ds) DNA oligonucleotides have recently been immobilized on gel pads of the same microarray to combine the advantages of cDNA and DNA oligonucleotide chips [63]. As illustrated in Fig. (15) abasic sites were generated on dsDNA by partial acidic depurination. Amino groups were then incorporated into the abasic sites upon reaction with ethylenediamine followed by reduction of the newly generated aldimines. Aminated DNA fragments were then condensed with arrayed aldehyde-

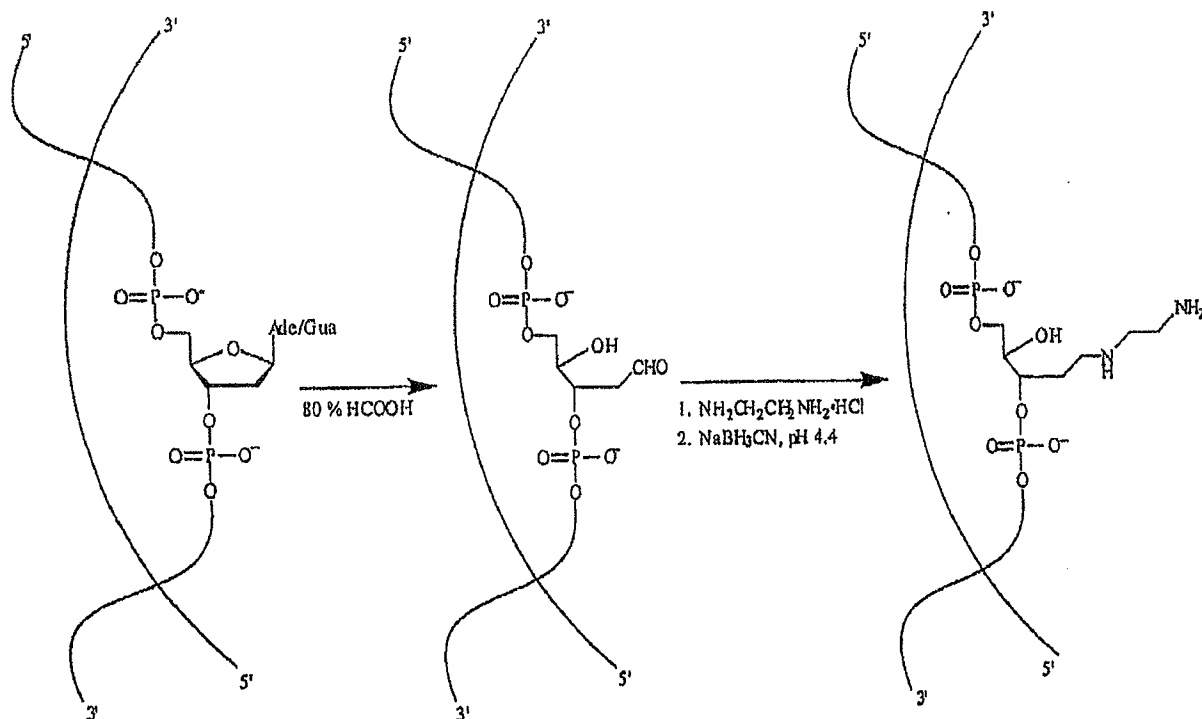


Fig. (15). Generation of abasic sites on dsDNA fragments to enable immobilization on gel pad microarrays.

polyacrylamide gel pads. Reduction of the resulting Schiff bases with pyridine-borane complex was performed in a manner similar to that presented in Fig. (14) for ssDNA oligonucleotides. Single-stranded and dsDNA fragments of 40 to 972 bp were immobilized on the gel pads of the DNA microchip [63]. The microchip could then be hybridized with fluorescently-labelled DNA-specific oligonucleotide probes. Interestingly, the generation of abasic sites on dsDNA and ssDNA according to Fig. (15) can alternatively be used for the incorporation of fluorescent markers into these biomolecules [64].

Planar supports other than glass can be used as arrayable surfaces for oligonucleotides. The use of polypyrrole films for this purpose will be the object of the next section.

IMMOBILIZATION OF OLIGONUCLEOTIDES ON POLYPYRROLE FILMS

A new methodology for the preparation of addressable DNA matrices relates to an electrochemical copolymerization of pyrrole with oligonucleotides bearing a pyrrole group at the 5'-terminus [65]. The preparation of pyrrole-oligonucleotide conjugates was accomplished as delineated in Fig. (16) by the addition of a pyrrole-containing deoxyribonucleoside phosphoramidite to the 5'-terminus of oligonucleotides according to standard solid-phase techniques. The coupling efficiency of the modified phosphoramidite was 90-95%. The copolymerization of pyrrole and pyrrole-oligonucleotide conjugates was performed on 1 cm² platinum electrodes in a classical

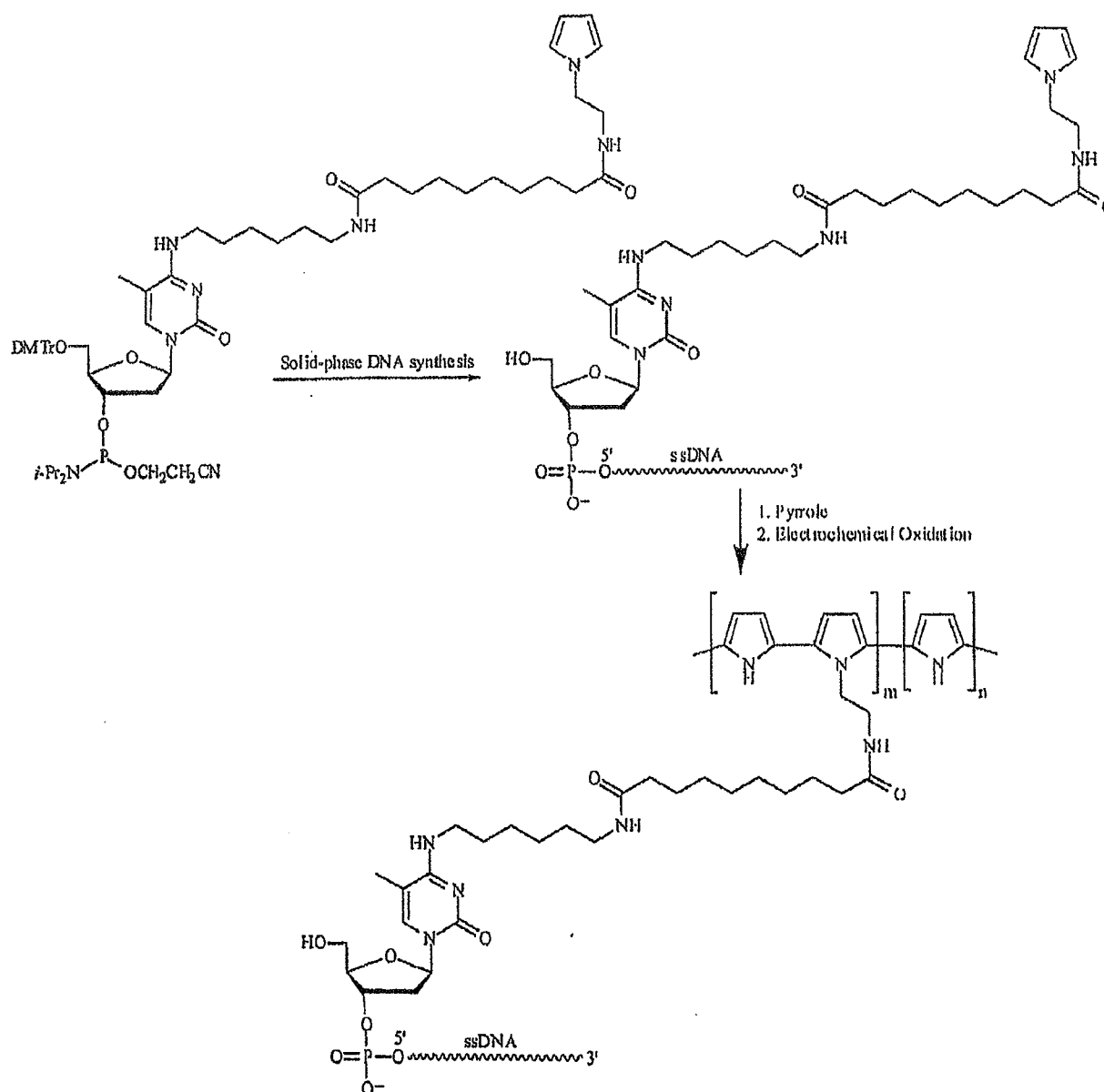


Fig. (16). Electropolymerization of pyrrole-oligonucleotide conjugates with pyrrole to form an oligonucleotide array on a polypyrrole film.

electrochemical cell [65]. The black insoluble film containing the oligonucleotides is electrically conducting so that its thickness depends on the current being used during polymerization. The density of oligonucleotide immobilization can also be controlled by the ratio of pyrrole-oligonucleotide conjugate/pyrrole being polymerized. The most reproducible results were obtained on 1 cm² electrodes when the concentration of pyrrole was 10 mM and that of the pyrrole-oligonucleotide conjugates was 1 μ M [65]. Oligonucleotides were stable during the electropolymerization process. The composition of the copolymer was estimated to be 1 pyrrole-oligonucleotide conjugate for 60,000 pyrrole units according to either coulometric measurements or by the use of 5'-[³²P]-labelled pyrrole-oligonucleotide conjugates [65].

Hybridization of oligonucleotides (13-mers) attached to a 20 nm thick polypyrrole film with 5'-[³²P]-labelled complementary oligonucleotides was complete in 1 h. No background for a non-complementary oligonucleotide was detected. The average oligonucleotide capture on a 20 nm polypyrrole film was *ca.* 2 pmol/cm². The accessibility of oligonucleotides linked to polypyrrole films for hybridization with a complementary oligonucleotides was estimated to be *ca.* 20%.

The chemical stability of polypyrrole films is very high; no degradation of the films was observed after more than 10 hybridization/stripping cycles. This methodology was extended to an oligonucleotide array (15-18 mers) constructed on a silicon support carrying either 48 or 128 gold microelectrodes (50 \times 50 μ m²) [66-68]. The ratio pyrrole-oligonucleotide conjugate/pyrrole was optimized by the use of 20 mM pyrrole and 0.6 μ M pyrrole-oligonucleotide conjugates during copolymerization. Thus, the ratio between pyrrole and pyrrole-oligonucleotide conjugates was 33,000 [66]. Under these conditions, the density of oligonucleotides (15-18 mers) attached to the film was *ca.* 200 fmol/mm² as estimated by the use of 5'-[³²P]-labelled complementary oligonucleotides. The DNA oligonucleotides attached to the polypyrrole films were specific for the hepatitis C virus (HCV) genus and for two common types of HCV, namely T1 and T2 [66]. Primers for PCR amplification of reverse-transcribed HCV RNAs were 5'-biotinylated. The biotinylated fragments were hybridized to the specific probes immobilized on the polypyrrole DNA chip for genotyping hepatitis C virus in blood samples. Hybrid formation was detected with a streptavidin-R-phycoerythrin conjugate [66-68] by epifluorescence microscopy. The insertion of a pentathymidine spacer between the HCV probe and the polypyrrole film led to increased hybridization signals with long DNA targets. Thus, steric considerations become important when relatively large DNA targets are interacting with complementary DNA probes that are attached very near the surface of the array [66].

Due to the facile preparation of polypyrrole films and their high chemical stability, the construction of DNA oligonucleotide arrays that could detect *ca.* 4800 nucleic acid molecules/ μ m² with a high degree of dimensional resolution is now readily accessible to many laboratories.

The synthesis of oligonucleotides on glass surfaces is another attractive approach to the preparation of DNA microarrays. This methodology will next be discussed.

OLIGONUCLEOTIDE SYNTHESIS ON A GLASS SURFACE

In an effort to analyze the hybridization behavior of a target DNA sequence with a complete set of complementary oligonucleotides, the synthesis of oligonucleotides on an arrayable glass surface has been described [69]. Specifically, glass plates were reacted with glycidioxypropyl trimethoxysilane and then heated with hexaethylene glycol to provide the hydroxylated glass surface pictured in Fig. (17) [69,70]. Deoxyribonucleoside phosphoramidites and standard reagents for solid-phase oligonucleotide synthesis were delivered to the surface of a functionalized glass plate through a teflon-sealed circular chamber that can manually be moved along the glass plate after each chain extension step. Final oligonucleotide deprotection was effected by incubation with 30% ammonium hydroxide in a pressure vessel constructed to accommodate glass plates.

The hexaethylene glycol linker was found not completely stable to ammonium hydroxide; only *ca.* 28% of the initial oligonucleotide loading remained after a 5 h treatment with 30% ammonium hydroxide at 55 °C. This loading corresponds to a surface area per oligonucleotide of *ca.* 39 Å². Such a linker instability can be attributed to the attachment site of the silane to the glass; cross-linking may be incomplete. This problem can be lessened by the use of base-ultralabile groups for nucleobase protection.

No hybridization signal was detectable when hexanucleotides were used as capture probes on the array. Hybridization signals were however observed when octanucleotides were employed for duplex formation [71].

In an experiment aimed at evaluating the effect of a sequence mismatch, a glass plate bearing both the dodecanucleotides 3'-CCCGCCGCTGCA and 3'-CCCGCCCTGCA was hybridized with 5'-[³²P]-GGGCGGCGACCT. Autoradiography showed that annealing occurred at low salt concentration (0.1 M NaCl) only with the fully complementary capture dodecanucleotide. No signal was detected with the mismatched sequence [70]. When using longer DNA and RNA sequences, the dominant factor in determining the extent of interaction between an arrayed oligonucleotide and these target sequences is prevention of duplex formation by intramolecular structures within the target sequences [69]. In this context, oligonucleotide arrays can be used to measure the potential of oligonucleotides for heteroduplex formation with mRNAs and provide a powerful tool for the selection of effective antisense reagents. Antisense technology for down-regulating the expression of any mRNA of known sequence has potential for therapy and the study of gene function. A "scanning" array of 1938 oligodeoxyribonucleotides ranging in length from monomers to 17-mers has been fabricated [72] on the surface of a glass plate according to the method described above [69-71]. The oligonucleotides were complementary to

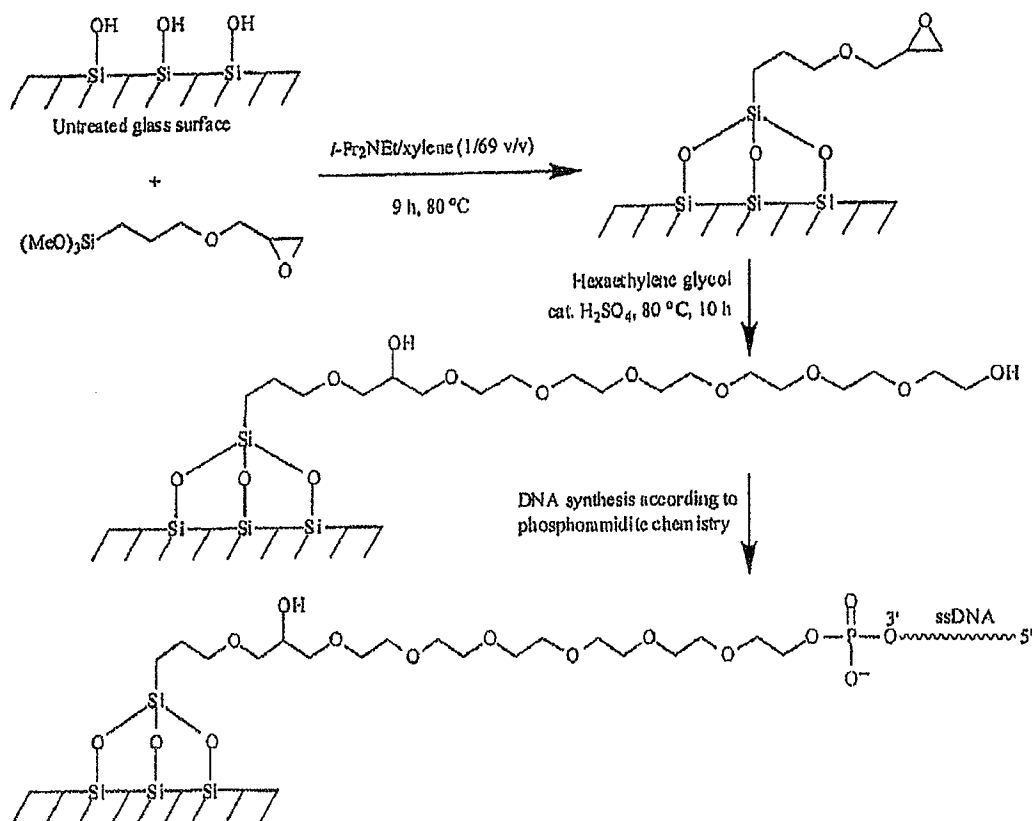


Fig. (17). Oligonucleotide synthesis performed directly on a glass surface.

the first 122 bases of rabbit β -globin mRNA comprising the 5'-UTR and bases 1 to 69 of the first exon. Intramolecular base-pairing of the mRNA target sequestered much of the sequence and thus prevented the intermolecular pairing essential to antisense activity. There was no detectable hybridization to bases 1 to 37 or 76 to 90 of the mRNA. The only sequence that gave a high duplex yield was that of an oligomer (15-mer) complementary to bases 46 to 60. This oligonucleotide was an effective antisense reagent in *in vitro* RNase H experiments; it inhibited β -globin synthesis by 50% at a concentration five-fold lower than any other oligonucleotide used in these studies [72]. No obvious features in the mRNA sequence or in its predicted secondary structure can justify the variation in heteroduplex yields. Along similar lines, the effects of structure on nucleic acid heteroduplex formation have been studied further by analyzing the hybridization of tRNA^{phe} to an entire set of complementary oligonucleotides ranging from single nucleotides to dodecanucleotides [73]. Interestingly, the most stable heteroduplexes were formed with both double-stranded as well as single-stranded regions. Heteroduplex formation was, however, disfavored by sharp turns or a lack of helical order in single-stranded regions, competition from bases displaced from a stem, and by stable tertiary interactions [73]. These results thus explain the variable success experienced by many in the selection of antisense oligonucleotides. It is important to mention that oligonucleotide arrays can be adapted to any analogue [74] that shows better affinity for mRNA targets to provide a

simple empirical method for the selection of effective antisense oligonucleotides.

Another approach to the synthesis of oligonucleotides on glass surfaces follows.

PHOTOLITHOGRAPHIC SYNTHESIS OF OLIGONUCLEOTIDES ON GLASS SURFACES

Photolithographic techniques have been applied to DNA sequence analysis by generating arrays of densely packed oligonucleotide probes on glass surfaces or DNA chips. Given that the sequence of each DNA probe is defined, DNA chips can be used to search for complementary sequences of

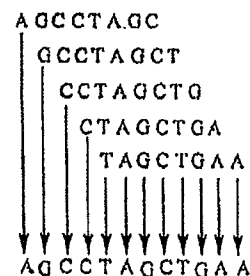
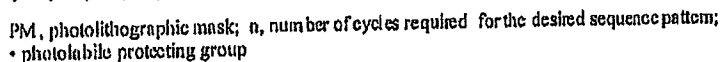
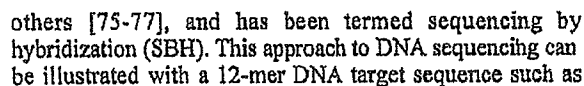


Fig. (18). Sequence determination of TCGATCGACTT by hybridization with a DNA chip containing a complete set of octanucleotide probes.



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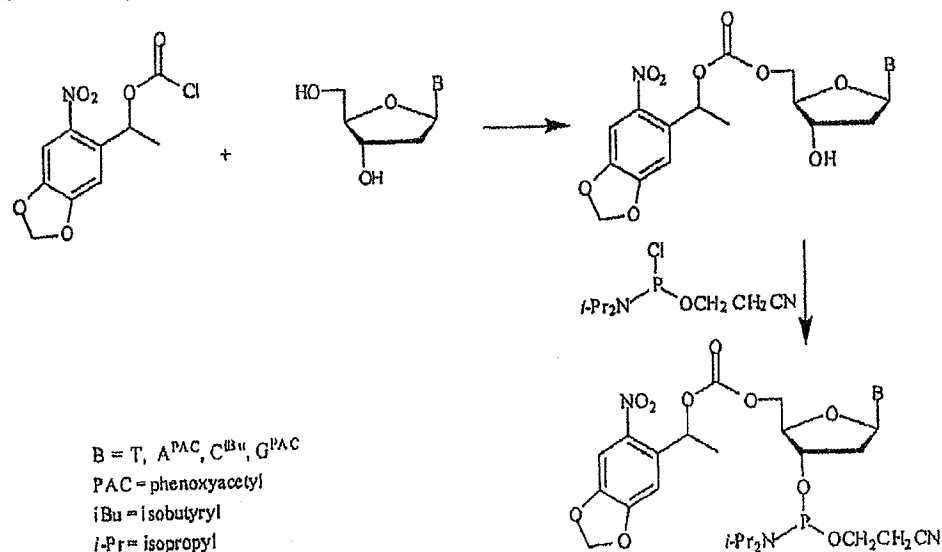


Fig. (21). Synthesis of 5'-photoprotected deoxyribonucleoside phosphoramidites for photolithographic preparation of oligonucleotides on glass surfaces.

TCGGATCGACTT. When this 12-mer is allowed to hybridize on a DNA chip containing a complete set of octanucleotide probes (65,536 octamers) only 5 of these probes will be perfectly complementary to the 12-mer DNA target. These are: AGCCTAGC, GCCTAGCT, CCTAGCTG, CTAGCTGA, and TAGCTGAA. Reconstruction of the sequence complementary to the original 12-mer DNA target can be achieved by aligning the overlapping sequences of the DNA chip probes as demonstrated in Fig. (18).

The glass surface used for covalently attaching oligonucleotides was first conditioned by treatment with concentrated sodium hydroxide. The surface was then functionalized with a solution of 10% bis(2-hydroxyethyl)aminopropyltriethoxysilane in ethanol. A linker was subsequently attached to the hydroxylated surface upon reaction with *O*-(4,4'-dimethoxytrityl) hexaethyloxy-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite as outlined in Fig. (19) [2,10].

The hydroxyl groups of the functionalized surface were photoprotected, and then the surface was exposed to UV light through a photolithographic mask to generate a pattern of free hydroxyl groups on the glass surface. These hydroxyls were reacted with 5'-photoprotected deoxyribonucleoside phosphoramidites according to standard solid-phase protocols to initiate chain extension. A second

photolithographic mask (PM) was then applied to the surface to generate another pattern of free hydroxyls, and cycles of phosphoramidite coupling and illumination were performed to achieve the desired pattern of oligonucleotide probes [2]. These steps are illustrated in Fig. (20).

The 5'-photolabile protecting group used for the deoxyribonucleoside phosphoramidites was the α -methyl-6-nitropiperonyloxycarbonyl group. The preparation of 5'-photoprotected deoxyribonucleoside phosphoramidites is presented in Fig. (21). The coupling efficiency of these phosphoramidites was slightly lower (92-94%) than that obtained with standard deoxyribonucleoside phosphoramidites [78]. The lower coupling efficiency was due to incomplete recovery of free 5'-hydroxyl groups after photolysis on the glass surface. Photodeprotection consisted of irradiation with 27.5 mW of 365 nm light per cm². Under these conditions, the rate of removal of the 5'-(α -methyl-6-nitropiperonyl)oxycarbonyl protecting group ($t_{1/2}$ = 12 s) was independent of either the nucleotide or the length of the growing oligomer. A moderate dependence on solvent polarity was noted with photolysis proceeding faster in the presence of non-polar solvents or in the absence of solvent ($t_{1/2}$ = 10-13 s at 27.5 mW/cm²) [78].

Unprotected 5'-hydroxyl groups associated with bound oligonucleotides can be quantitated by labeling with a fluorescent phosphoramidite derivative like the one shown in

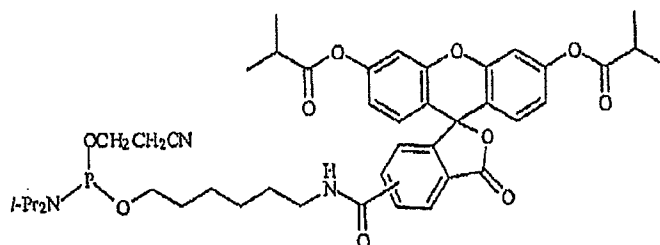


Fig. (22). Fluorescent phosphoramidite derivative useful for the determination of oligonucleotide surface density on glass.

Fig. (22) and measuring surface fluorescence by confocal microscopy [78].

To demonstrate the sequence specificity of hybridization probes on the DNA chip, combinatorial probes were targeted by a fluorescently-labelled octanucleotide. It was discovered that the labelled oligonucleotide hybridized specifically to its complementary sequence with a fluorescence signal intensity 5- to 35-fold stronger than that obtained with single or double base-pair mismatches [2].

The use of light-directed DNA synthesis with photoprotected phosphoramidite monomers can achieve densities of *ca.* 10^6 sequences/cm² on the DNA chip. The 3',5'-dimethoxybenzoin carbonate group is another 5'-photolabile group for deoxyribonucleoside phosphoramidites that has been developed to optimize photolithographic DNA synthesis on glass surfaces. Fig. (23) shows the detailed preparation of these phosphoramidites [79,80]. The coupling efficiency of these phosphoramidites in the synthesis of homopolymers up to dodecamers was: 91-98% for poly-T; 82-95% for poly-C; 79-92% for poly-A and 74-84% for poly-G [80]. Photochemical deprotection rates of 5'-(3',5'-dimethoxybenzoin)carbonyl-protected nucleosides on glass surfaces depended on irradiation wavelengths and solvent polarity. Photodeprotection occurred faster at 310 nm ($t_{1/2}$ = 5.5 to 13 sec) than at 365 nm ($t_{1/2}$ = 5.6 to 17 sec) in a non-polar solvent or without solvent [80]. The 5'-(3',5'-dimethoxybenzoin)carbonyl-protected deoxyribonucleoside phosphoramidites were used to prepare an array of the sequence 5'-AANTANCTAC where N is A, C, G, or T. The array was then hybridized with the fluorescently labelled 5'-CTGAACGGTAGCATCTTGAC. Surface fluorescence

imaging indicated sequence-specific hybridization with an average signal to background ratio greater than 6 [80]. These results compare well in terms of hybridization fidelity to those obtained earlier from an array of oligonucleotides prepared from 5'-(1"-methyl-6"-nitropiperonyloxy)carbonyl-protected deoxyribo-nucleoside phosphoramidites [2].

Photolabile 2-(2-nitrophenyl)ethoxycarbonyl groups have recently been applied to the preparation of the deoxyribonucleoside and ribonucleoside phosphoramidites depicted in Fig. (24). These phosphoramidites have already been used in the construction of oligonucleotide arrays and have shown promising properties [81]. Upon UV irradiation at 365 nm in aqueous methanol, the 5'-O-2-(2-nitrophenyl)propoxycarbonyl group was released from the 5'-protected thymidine with a half-life of 40-60 sec. Thymidine was recovered in a yield not exceeding 72%. Under similar conditions 5'-O-(1"-methyl-6"-nitropiperonyloxy)carbonyl-protected thymidine was photodeprotected at a similar rate in dioxan. However, thymidine was recovered in 82% yield [81]. In both cases, secondary photoreactions may account for the incomplete recovery of thymidine. Thus, problems inherent to photochemical deprotection may affect the absolute yield and quality of DNA oligonucleotides synthesized according to photolithographic methods on arrays. In this regard, it has recently been demonstrated that irradiation of 5'-O-phenacyloxycarbonyl thymidine at 390 nm in the presence of 9,10-dimethylanthracene cleanly produced thymidine in 91% yield [82]. This electron-transfer mediated photochemical release of alcohols from phenacyl carbonate esters may lead to improvements in the photolithographic preparation of DNA oligonucleotide arrays. One of the major drawbacks in the fabrication of

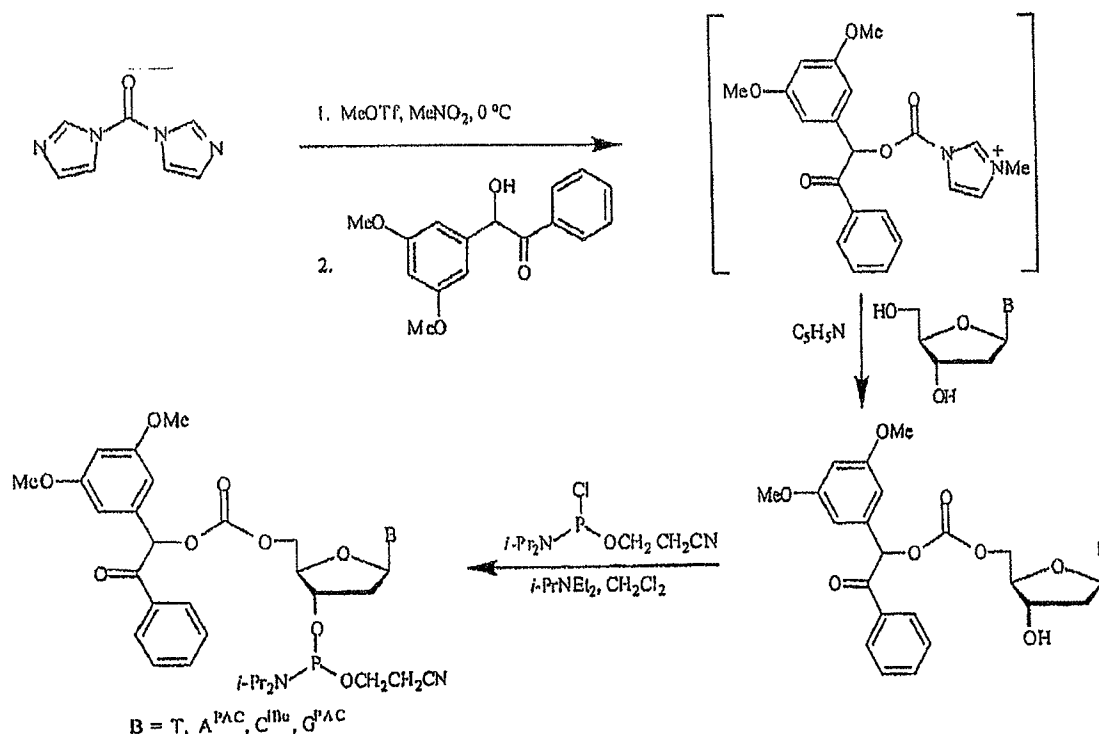


Fig. (23). Preparation of 5'-photoprotected deoxyribonucleoside phosphoramidites for photolithographic synthesis of oligonucleotides on glass surfaces.

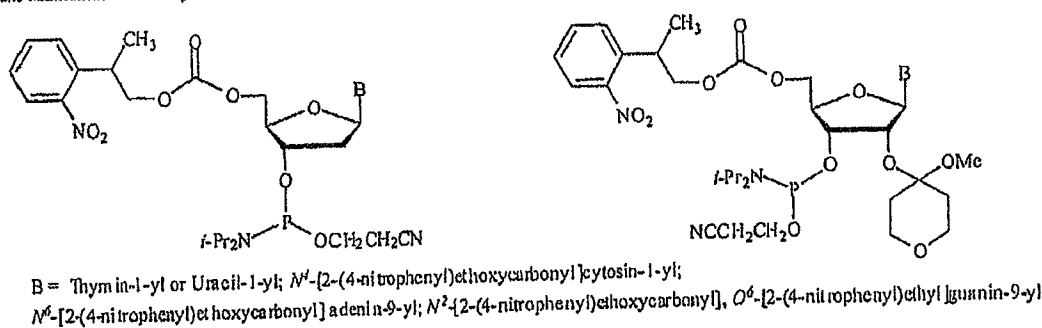


Fig. (24). Nucleoside phosphoramidites functionalized with a 5'-photolabile protecting group.

oligonucleotide microarrays according to photolithographic techniques is the requirement for expensive masks used to control light-directed oligonucleotide synthesis. For example, the preparation of a DNA microarray may require 100 different masks leading to high cost and long fabrication time. A maskless technique for light-directed synthesis of high-resolution microarrays that uses a digital micromirror array to form computer-generated masks has been engineered

[83]. A 1:1 reflective imaging system forms an ultraviolet image of the virtual mask on the glass surface which is mounted in a flow cell reaction chamber connected to a DNA synthesizer. This technology has been used to synthesize oligonucleotide microarrays with high repetitive coupling efficiencies (*ca.* 95% in the synthesis of 18-mers). These arrays could, after hybridization, readily discriminate single-base pair mismatches [83].

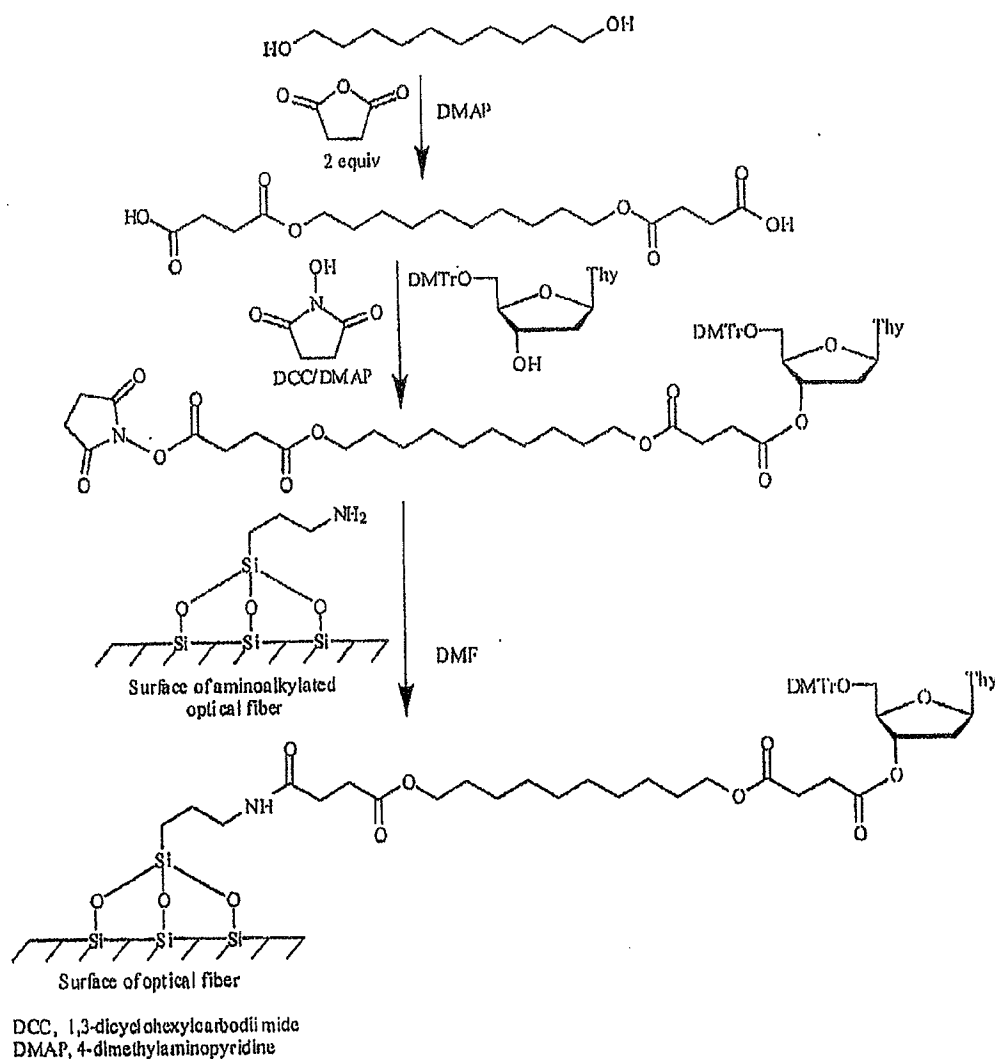


Fig. (25). Surface functionalization of optical fibers to permit direct oligonucleotide synthesis.

Aside from planar glass surfaces, oligonucleotides have been covalently immobilized or synthesized directly onto optical fibers. This method for DNA hybridization analysis will be the next topic of this review.

IMMOBILIZATION OF DNA ONTO OPTICAL FIBERS

The application of optical fibers and fluorescence detection systems to the development of biosensors for direct analysis of DNA hybridization events has over the years attracted considerable attention. Like any other glass surfaces, optical fibers are functionalized by treatment with 0.5% 3-aminopropyltriethoxysilane in dry toluene. The attachment of a nucleoside to the surface of aminosilanized optical fibers was accomplished according to Fig. (25). Specifically, 1,10-decanediol was reacted with succinic anhydride to the corresponding bis-hemisuccinate ester which was condensed with *N*-hydroxysuccinimide and 5'-*O*-(4,4'-dimethoxytrityl) thymidine in the presence of *N,N*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) [84,85]. This reaction produced a nucleoside functionalized with an activated 3'-*O*-alkylester which in turn was reacted with the aminoalkyl groups on the surface of the optical fibers. A chamber (0.2 μ mole DNA synthesis column) filled with nucleoside-functionalized optical fibers and inert material was subjected to standard 2-cyanoethyl phosphoramidite solid-phase DNA

synthesis protocols to achieve the preparation of dT₂₀. The 2-cyanoethyl phosphate blocking groups were removed by treatment with triethylamine in acetonitrile. The covalently immobilized dT₂₀-oligomer formed a dsDNA duplex with added complementary dA₂₀. After ethidium bromide staining, the duplex was detected by fluorescence microscopy [84,85]. The detection limit of the optic fiber biosensor was 86 ng/mL of complementary ssDNA with a hybridization analysis time of 46 min [84,85]. The sensor was reusable and retained full activity after prolonged storage (1 year), harsh washing conditions (sonication), and autoclaving. The extent of hybridization between the immobilized and complementary nucleic acid strands was determined by thermal denaturation experiments; all 20 bases of each strand were found to participate in duplex formation [85].

The functionalization of optical fibers can be performed differently. For example, treatment of clean optical fibers with a solution of 3-glycidopropyltrimethoxysilane, *N,N*-diisopropylethylamine and xylene (30:1:100 v/v/v) at 80 °C followed by mono-(4,4'-dimethoxytrityl) hexaethylene glycol in the presence of catalytic amounts of sodium hydride at 40°C afforded, after capping and detritylation, fibers functionalized with ~1 nmole of hydroxy group/fiber (see Fig. (26)) [86]. Optical fibers can thus be packed into a DNA synthesis column with ~1 nmole of hydroxy group/fiber (see Fig. (26)) [86]. Optical fibers can thus be packed into a DNA synthesis column and DNA (in this case dA₁₀) can be grown on the surface of each optical fiber

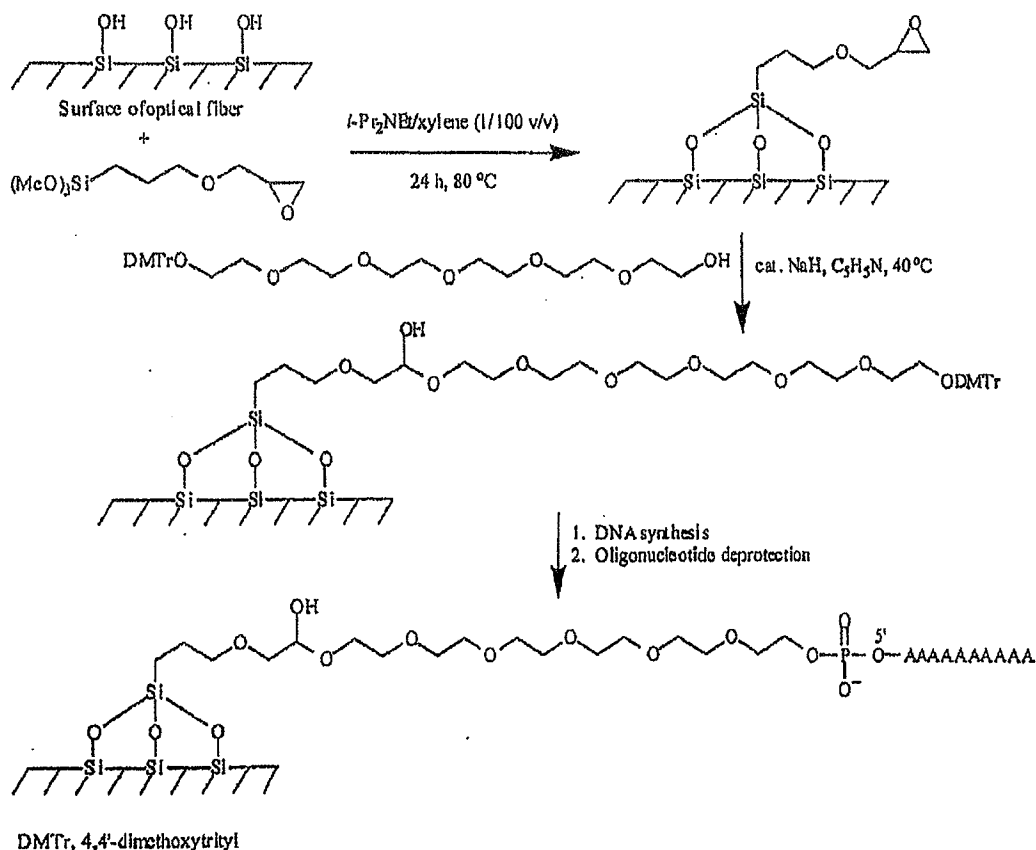


Fig. (26). Alternate functionalization of optical fibers to enable direct oligonucleotide synthesis.

according to standard solid-phase protocols. After oligonucleotide deprotection, fluorescence studies showed unequivocal hybridization between dA₁₀ immobilized on the fibers and complementary dT₁₀ supplied in solution. Although fluorescence was produced by intercalated ethidium bromide, changes in fluorescence intensity permitted the detection of triplex formation (TAT pyrimidine motif) [86].

Another example of optical fiber functionalization pertained to the conversion of 12-nitrododecanoic acid to its acyl chloride followed by condensation with 3-aminopropyltriethoxysilane to give the corresponding nitroalkylated silane which was purified by vacuum distillation. Hydrogen peroxide-treated optical fibers were then immersed in a solution of 4% nitroalkylated silane in toluene. The nitro function of the nitroalkylated fiber surface was reduced to an amine in a solution of 6 M hydrochloric acid to which was added zinc dust [87]. Pre-synthesized oligonucleotides carrying a 5'-phosphate monoester (such as pT₂₀) were then coupled to those optical fibers functionalized with long aminoalkylated spacers in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-methylimidazole at 50 °C. Hybridization of synthetic dA₂₀ to dT₂₀ immobilized on the fiber surface was detected by the fluorescence of intercalated ethidium bromide. It was found that this method for attaching oligonucleotides onto optical fibers was less efficient (by a factor of 4) than the direct oligonucleotide synthesis method [87]. Using a similar strategy, 5'-aminoalkylated oligonucleotides have been covalently attached to one of the terminal tips (200 μ m in diameter) of optical fibers. A bundle of optical fibers was then assembled with each fiber carrying a different oligonucleotide probe [88]. Hybridization of fluorescently-labelled complementary oligonucleotides to the fiber-optic biosensor array was monitored by observing the increase in fluorescence that resulted from DNA oligonucleotide binding. This approach permitted the fast (less than 10 min) and sensitive (10 nM) detection of multiple DNA sequences simultaneously [88].

5'-Aminoalkylated oligonucleotides have also been covalently linked to the surface of a tapered optical fiber that had previously been treated with 3-aminopropyltriethoxysilane and glutaraldehyde [89]. Real-time hybridizations have been carried out using synthetic oligonucleotides labelled at the 5'-terminus with the near-

infrared fluorophore IRD 41 and complementary oligonucleotides conjugated to the tapered fiber surface. Considering that the excitation and emission wavelengths of IRD 41 are 787 nm and 807 nm, respectively, the Near-IR spectral region is particularly desirable for examining clinical and other biological samples; naturally occurring biological materials show no emission of fluorescence when submitted to electromagnetic radiation in this region of the spectrum. Thus, the tapered optical fiber hybridization assay sensor, which relied on the evanescent field excitation of fluorescence from surface-bound fluorophores, was sufficiently sensitive to detect hybridization when as little as 70 pM IRD-41-labelled oligonucleotides was present in the hybridization medium [89].

A polypropylene surface is another type of support that is amenable to the preparation of DNA microarrays. The synthesis of oligonucleotides on polypropylene sheets is examined in the next and last section of this report.

COVALENT IMMOBILIZATION OF OLIGONUCLEOTIDES ON POLYPROPYLENE SURFACES

Polypropylene has been modified to serve as a new solid-phase support for oligonucleotide synthesis. Typically, polypropylene membranes were aminated upon exposure to an ammonia plasma generated by radiofrequency plasma discharge. The amine content of aminated polypropylene was quantitated by first reacting the surface with sulfo-succinimidyl 4-*O*-(4,4'-dimethoxytrityl)butyrate, and then, by releasing and measuring the dimethoxytrityl cation under acidic conditions. The amine content was determined to be 20 ± 4.5 nmol/cm² [90]. The synthesis of oligonucleotides on aminated polypropylene surfaces was carried out as described in Fig. (27) using phosphoramidite-based protocols. Oligonucleotide density on polypropylene has been estimated as 10.2 ± 1.4 nmol/cm² [90]. A high-density oligonucleotide arrays composed of 4096 oligonucleotides (8- to 16-mers) was constructed to conduct hybridization with a biotinylated oligonucleotide target (18-mer) complementary to the F508 codon region of the cystic fibrosis transmembrane conductance regulator gene. DNA-DNA hybrids were detected using fluorescently-labelled streptavidin, and thus clearly demonstrated that

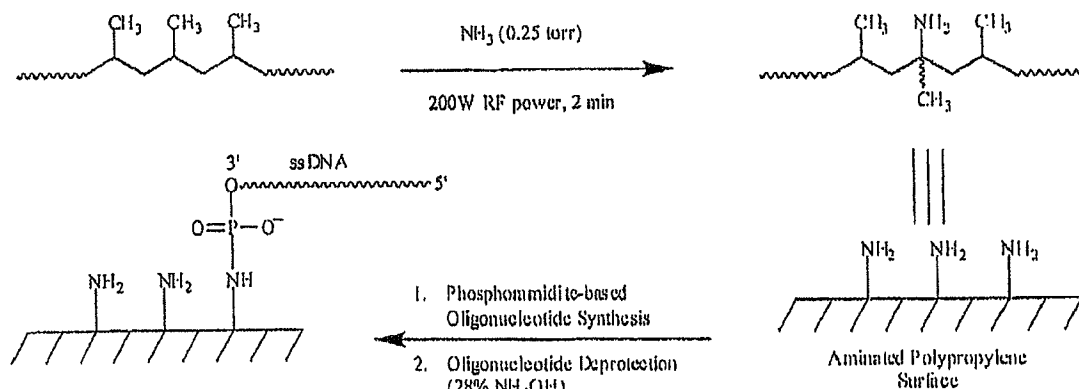
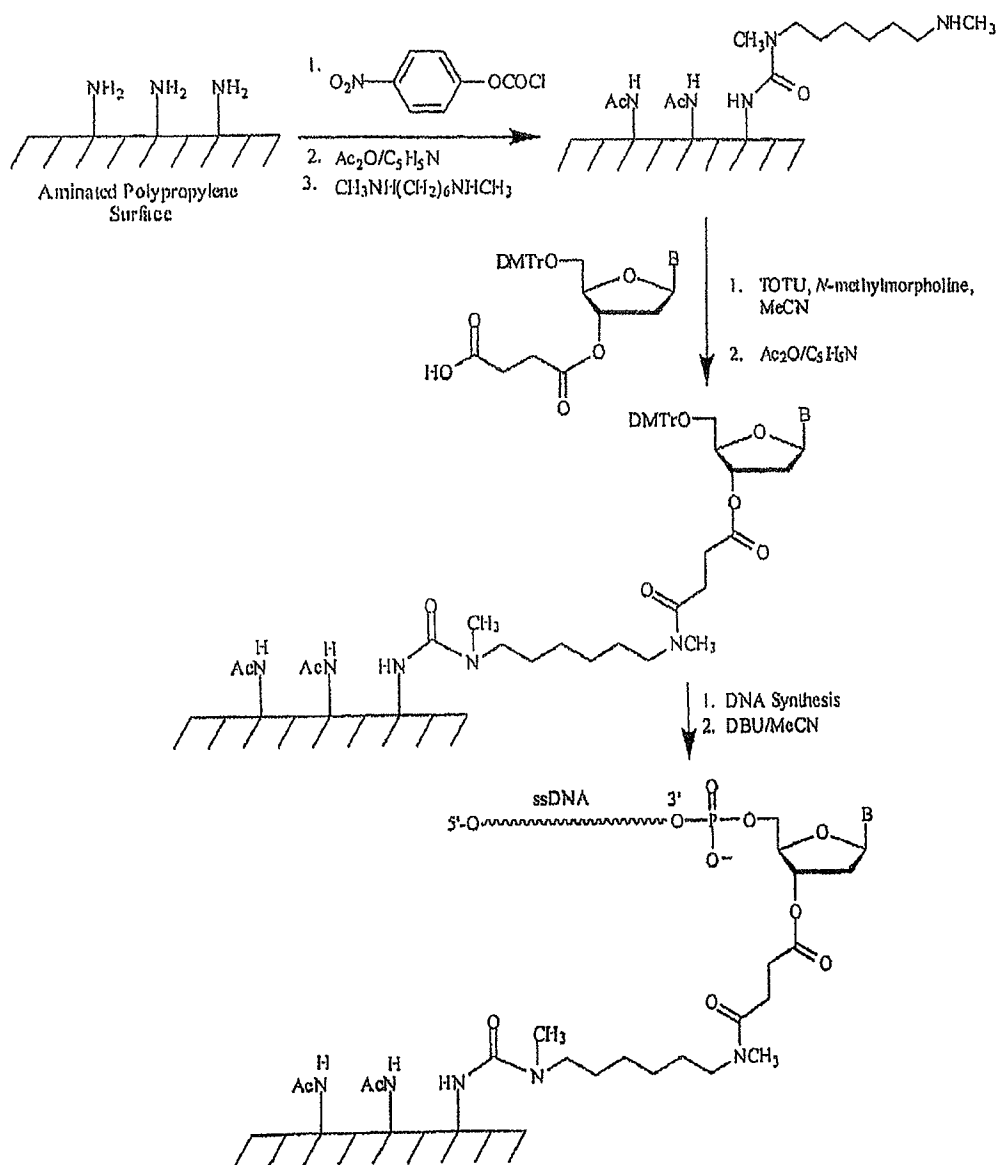


Fig. (27). Synthesis of oligonucleotides on aminated polypropylene surfaces.

oligonucleotide arrays can be generated from aminated polypropylene surfaces [91].

Given the availability of aminated polypropylene sheets, these can easily be functionalized by treatment with 4-nitrophenyl chloroformate and triethylamine in dichloromethane/dioxane (1:1 v/v). After reacting unreacted amino groups with acetic anhydride, the activated polypropylene surface was treated with 1,6-bis-(methylamino)hexane in acetonitrile or DMF at 40 °C, and then with the 3'-*O*-succinate derivatives of protected nucleosides in the presence of *N*-methylmorpholine and *O*-[ethoxycarbonyl]cyanomethylenamino]-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TOTU) [92]. Synthesis

of oligonucleotides on polypropylene surface was achieved according to phosphoramidite chemistry as displayed in Fig. (28). Oligonucleotides were deprotected by treatment with DBU without being released from the polypropylene surface. The purity of these oligonucleotides can nonetheless be analyzed by capillary electrophoresis after ammonolytic cleavage from the polypropylene surface. Given the purity of full-length oligonucleotides, the coupling efficiency of deoxyribonucleoside phosphoramidites on polypropylene was estimated to be greater than 98.5% and the amount of oligonucleotides being released was estimated to be more than 25 pmol/cm². Hybridization experiments were performed using 5'-radiolabeled oligonucleotides complementary to those oligonucleotides covalently



B = dA^{NIPHC}; dC^{NIPHC/NIPHC}; dC^{NIPHC}; dT; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene
TCTU = O-[(ethoxycarbonyl)cyanomethyl]enamine-*N,N,N',N'*-tetramethyluronium tetrafluoroborate

Fig. (28). Functionalization of aminated polypropylene surfaces to conduct oligonucleotide synthesis.

The covalent attachment of oligonucleotides to a solid surface generates problems that are not encountered in homogenous solutions, as it constrains the ways

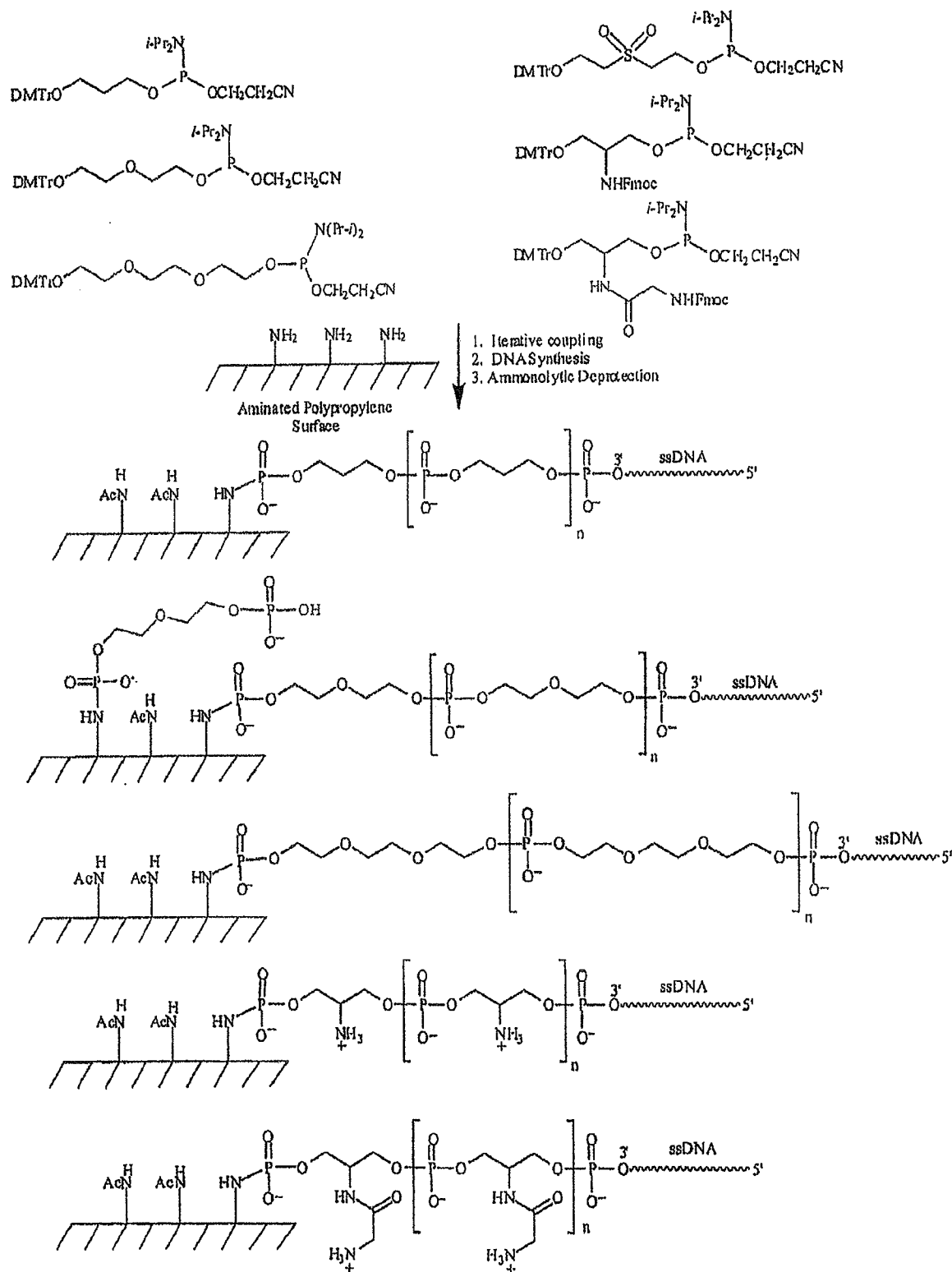


Fig. (29). Synthesis of oligonucleotides on aminated polypropylene surfaces functionalized with various types of linkers.

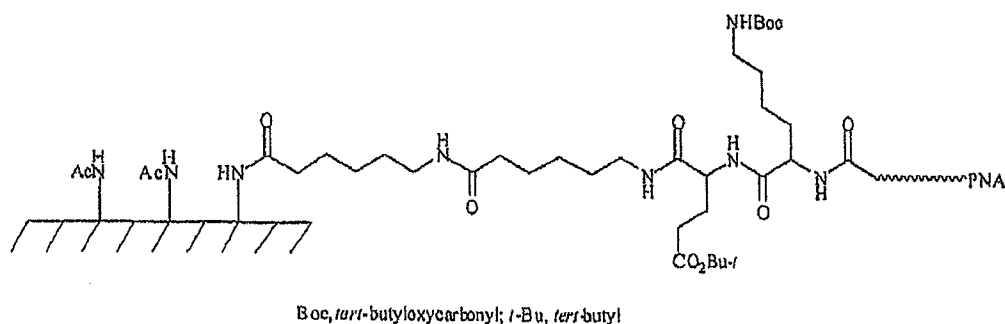


Fig. (30). Synthesis of PNA oligomers on aminated polypropylene surfaces derivatized with a peptide-spacer combination.

oligonucleotides can interact with each other. Complementary oligonucleotides in solution may be prevented from making a close approach to the surface-bound oligonucleotides unless there is sufficient distance between the oligonucleotides and the surface. In addition, the surface density of bound oligonucleotides may hinder the approach of complementary oligonucleotides in solution. Finally, the nature of the surface to which oligonucleotides are attached, its charge, hydrophobicity, and degree of solvation are all likely to influence the environment wherein oligonucleotide interactions will take place. In an attempt to study these parameters, the incorporation of various spacers between oligonucleotides and the surface to which they are attached to has been performed by the use of the phosphoramidites described in Fig. (29). It has been demonstrated that the best spacer for oligonucleotides covalently linked to polypropylene should have low negative charge density and have a length of 30-60 atoms to produce maximum hybridization yields. Although the presence of both positively and negatively charged groups in the spacer decreased the yield of hybridization, the nature of the spacer should be amphiphilic to provide hydrophilic properties. The influence of oligonucleotide surface density on hybridization properties was examined by the use of a combination of cleavable and stable linkers. The highest hybridization yields were obtained for surfaces containing ~50% of the maximum oligonucleotide concentration [93].

Noteworthy is the use of aminated membranes in the preparation of PNA oligomer arrays (Fig. (30)). The aminated membrane was first derivatized with the peptide spacer glutamic acid-(γ -*tert*-butyl ester)-(ϵ -amino hexanoic acid)-(ϵ -amino hexanoic acid) using standard Fmoc-chemistry [94]. The peptide-functionalized membrane was then spotted, robotically, with activated Fmoc-lysine-(ϵ -*tert*-butoxycarbonyl). After 30 min, the membrane was treated with 5% acetic anhydride in *N*-methyl-2-pyrrolidone to cap all amino groups outside the spotted areas. PNA synthesis was performed using robotic deposition of a mixture of Fmoc-protected PNA monomers, diisopropylcarbodiimide and 1-hydroxyazabenzotriazole. The coupling reaction time was 20 min. Arrays of either 384 or 576 spots were synthesized. The area of each spot was ~0.2 cm² with a final loading of ~10 nmoles crude PNA oligomers (up to 16-mers). The average coupling yield was 85-96% according to a colorimetric assay. Upon completion of the synthesis process, the PNA oligomers were side-chain deprotected by

immersing the dry membrane in a mixture of 90% TFA, 5% water, and 5% triethylsilane [94]. Hybridization experiments were carried out with 5'-[³²P]-DNA oligonucleotides. The attachment linkage of the PNA oligomers to the membrane was found to be very stable. No apparent decrease in signal intensity could be detected even after 30 successive hybridization reactions. Hybridizations followed the Watson-Crick base-pairing rules and were characterized by higher duplex stabilities (1-1.5 °C/base pair) than those measured on the corresponding DNA oligonucleotide array. Both the affinity and specificity of DNA hybridization with immobilized PNA oligomers depended on hybridization conditions. Discrimination between hybridization to full complementary PNA sequences and truncated or mismatched ones was possible at low salt concentrations (*ca.* 10 nM); the presence of *N*-lauroylsarcosine was however required for discriminative hybridizations. It should be noted that exceptionally strong and entirely unspecific complexes on G-rich PNA sequences occurred at a salt concentration below 10 nM. It is still unclear why these complexes exhibited such a stability [94]. Finally, the application of PNA arrays to the detection of single point mutations in DNA analytes has recently been reported to add to the repertoire of methods for genomic analysis [95].

CONCLUDING REMARKS

DNA microarray technology is a powerful tool in providing a better understanding of gene expression analyses and in identifying genomic polymorphisms. However, it seems clear that this technology can benefit from fundamental studies on: (i) how nucleic acid sequence affects hybrid formation and stability; (ii) how nucleic acid probes are best attached to the arrays; (iii) how long a spacer between the probes and the surface of the arrays needs to be for optimal hybridization reactions; (iv) the determination of ideal oligonucleotide attachment density on the arrays; and (v) how hybridization kinetics and specificity can be improved. This review did indeed attempt to provide answers to these issues in order to facilitate the correct interpretation of the massive amounts of data that can be generated from DNA microarrays. A greater insight into cellular processes and an improved knowledge of the genetic variations in the human genome shall emerge from increasingly refined microarray technologies.

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